(54) Title: METHODS AND COMPOSITIONS FOR MODULATION OF WOUND HEALING

(57) Abstract

To achieve cellular selectivity of modulation of wound healing, this invention provides conjugates of cell-proliferation-modulating agents and macromolecules that bind with high affinity to extracellular matrix components such as collagen, fibronectin and proteoglycans and/or a specific cell type that migrates into a wound site. These conjugates restrict the proliferation-modulating agent to the wound site and additionally increase the residence time of the conjugate at that site. Use of the conjugate reduces side effects due to the proliferation-modulating agent and reduces frequency of administration.

METHODS AND COMPOSITIONS FOR MODULATION
OF WOUND HEALING

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of USSN 08/258,550, filed June 9, 1994, which disclosure is incorporated herein by reference.

INTRODUCTION

Technical Field

This invention relates to methods and compositions for modulating wound healing. The invention is exemplified by a composition comprising a conjugate of a tissue extracellular matrix-specific binding molecule coupled to an antiproliferative or cytotoxic agent for use in inhibition of wound healing in the eye as an adjunct to glaucoma filtering surgery for use in the treatment of proliferative vitreoretinopathy.

Background

Glaucoma encompasses a heterogeneous group of eye diseases characterized by a classical triad of symptoms: elevated intraocular pressure (IOP), optic nerve damage, and progressive visual field loss. These eye disorders affect more than two million Americans and are a leading cause of blindness in the United States. The increase in IOP is due to a decrease in the outflow of aqueous humor, the fluid in the anterior segment of the eye that is responsible for maintaining pressure balance for the entire eye. Glaucomas can be classified according to etiology (primary, secondary or developmental) or to the state of the iridocorneal angle (open or closed). The primary glaucomas are open-angle glaucoma, low-tension glaucoma, and single-closure glaucoma. Secondary glaucomas are the result of a defined local or systemic process which causes elevated IOP, i.e., pigmentary, neovascular, pseudoexfoliative, steroid-induced or lens-induced glaucomas and glaucomas secondary to ocular tumors, trauma, inflammation, hemorrhage and surgery. Developmental glaucomas arise from congenital abnormalities, including developmental anomalies of the anterior

chamber angle. The dominant form of glaucoma in the United States and Europe is primary open-angle glaucoma (POAG).

Current medical therapy for glaucoma involves medical, laser and conventional surgical approaches and is directed toward reduction in IOP to a level
5   that is presumed to arrest the progressive damage to the optic nerve. Medical therapy of POAG involves treatment with cholinergics (pilocarpine) and/or adrengerics (epinephrine, timolol), as well as oral therapy with carbonic anhydrase inhibitors (acetazolamide). While most glaucoma patients initially respond to drug therapy, many become refractory over time. For those individuals, maintenance of normal
10   IOP requires surgical intervention.

Surgical techniques include laser trabeculoplasty and various types of glaucoma filtering surgery. Laser trabeculoplasty involves the controlled application of intense light to the trabecular meshwork in order to shrink its collagenous tissue and thereby open drainage channels for the aqueous humor. In most patients, laser
15   trabeculoplasty reduces IOP by approximately 25 percent and is often used as an adjunct to medical therapy before resorting to more invasive surgical procedures. However, the initial decreases in IOP observed following trabeculoplasty are often lost after a few months or years.

Glaucoma filtering surgery (GFS) is performed to create a drainage channel
20   for aqueous humor outflow from the anterior chamber in order to lower IOP. Although glaucoma filtering surgery has the potential to replace drug therapy permanently, its current high failure rate makes it an unattractive choice. Uncontrolled scarring of the surgical site usually occurs and the scar tissue frequently blocks the newly created drainage channel. For this reason, GFS is used primarily
25   on "end stage" glaucoma patients who no longer respond to drug therapy and therefore have no other options. Most successful glaucoma filtering surgery results in the creation of a filtering bleb, which is an elevation of the conjunctiva at the surgical site. Numerous techniques are sometimes employed as modification to maintain the patency of the drainage fistula, including the use of biocompatible plastic tubes or
30   valves, yet scarring over of the drainage channel frequently causes blockage of the fistula and concomitant increase in IOP. *See* Shields, Ch. 36, "Filtering Surgery" Textbook of Glaucoma, 3rd ed., Williams and Wilkins, Baltimore, 1992, .

Recent clinical studies have demonstrated that introduction into the surgical wound site of agents which inhibit the healing process can improve the success rate of GFS. However, these agents generally are administered by non-specific means such as application by sponge to the drainage filter tissue during the surgical procedure or
5   by repeated, painful injections into the conjunctiva after the operation. These agents also have undesirable side effects due to their inherent toxicity and the lack of specific localization to the surgical site. Further, the use of antiproliferative agents such as mitomycin C and 5-FU applied directly to the surgical site, often results in inadequate wound healing, leakage of the aqueous humor, hypotony or very low
10   pressure leading to further complications.

Another ophthalmic disease related to harmful proliferation of cells at a surgical site is proliferative vitreoretinopathy (PVR), a potentially blinding disease caused by the escape into and subsequent proliferation of retinal pigmented epithelial cells and other cell types in the vitreous cavity (posterior segment of the eye). This
15   growth and differentiation of cells with the accompanying formation of an epiretinal membrane can eventually lead to a tractional distortion of the retina and eventually detachment of the retina. PVR may occur as a result of accidental or surgical trauma. PVR is currently treated surgically with a procedure called vitrectomy. The operation consists of inserting the tip of a small cutting instrument into the vitreous
20   and removing the cloudy vitreous and scar tissue resulting from the trauma. Due to the delicate nature of the vitrectomy, a typical procedure takes two to three hours to complete. Pharmacological approaches investigated for the treatment or prevention of PVR include steroids and anti-proliferative drugs. These agents are intended to inhibit the initial macrophage-mediated inflammation, which is part of the wound-
25   healing process, in traumatic PVR or to control the proliferation process by killing the respective cell types (RPE, fibroblasts) in a non-specific manner. This experimental therapy has been hindered primarily by non-specific toxicity and the need for multiple administration of the drugs due to their short half-life.

For both glaucoma filtering surgery and proliferative vitreoretinopathy, it
30   would be of interest for the treatment and or prevention of these disorders to identify new methods and compositions to provide localization of agents capable of modulating wound healing at the surgical site.

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Relevant Literature

The use of colchicine to inhibit cell migration and proliferation *in vitro* (Lemor, *et al.* (1986) *Arch. Ophthalmol* 104:1223) and to treat PVR in an *in vivo* animal model (Lemor and Glaser (1986) *Arch. Ophthalmol.* 104:1226) has been

5   reported. The use of minoxidil (Handa, *et al.* (1983) *Invest. Ophthalmol. Vis. Sci.* 34:567-575) and retinoic acid have been reported in experimental models of PVR (Ver Straeten, *et al.* (1990) *Invest. Ophthalmol. Vis. Sci.* 31:1761-1766). Hajek, *et al.* ((1986) *Invest. Ophthalmol. Vis. Sci.* 37:1010) investigated the pharmacologic inhibition of cell proliferation and migration *in vitro*.

10   Bi-specific antibodies for use in immunodiagnostic reagents or in the targeting of cytotoxic lymphocytes, drugs or enzymes to specific targets such as cancer cells have been described. *See* Suresh, *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:7989-7993; Runge, *et al.* (1990) *Bioconjugate Chem.* 1:274-277; Foglesong, *et al.* (1989) *Cancer Immunol. Immunother.* 30:177-184); and Milstein and Cuello (1984)

15   *Immunol. Today* 5:299-304). MacKay, *et al.* (1993) have reported on the construction of a fusion protein with epidermal growth factor (EGF) and the collagen-binding domain of fibronectin.

The following references may also be relevant to the subject invention: Hudecz *et al.* (1990) *Bioconjugate Chem.*, 1:197-204; Wei-Chiang *et al.* (1981)

20   *Biochem. Biophys. Res. Commun.*, 102(3):1048-1054; Yang *et al.* (1988) *Proc. Natl. Acad. Sci. U.S.A.*, 85:1189-1193; Wilson *et al.* (1991) *J. of Ocular Pharm.*, 7(1):1-8; Xu *et al.* (1993) *Ophthal. Surgery*, 24(6):382-388; Tahery *et al.* (1989) *J. of Ocular Pharm.*, 5(2):155-179; Sherwood *et al.* (1993) *J. of Glaucoma*, 2:64-67; Jampel *et al.* (1993) *J. of Glaucoma*, 2:58-63; Palmer (1991) *Ophthalmology*,

25   98:317-321; Miller *et al.* (1989) *Ophthal. Surgery*, 20(5):350-357; European Patent Application 0 253 202 A2; European Patent Application 0 299 467 A1; Miller *et al.* (1991) *Invest. Ophthal. Vis. Sci.*, 33(4):1160; Wilkie *et al.* (1991) *Am. J. Vet. Res.*, V52, N3:441-444; King *et al.* (1991) *American Journal of Veterinary Research*, V52, N12:2067-2070; Potter *et al.* (1988) *Journal of Ocular Pharmacology*, 4(1):19-

30   28;Brooks *et al.* (1990) *Small Animal Ophthalmology*, 20:775-797; M. Bruce Shields, Textbook of Glaucoma, 3rd ed., 1992., Ch. 34 and 36.

The following references relate to hyaluronic acid (also called hyaluronan): Goetinck *et al.* (1987) *J. Cell Biol.*, 105:2403-2408; Geng *et al.* (1994) *Invest.*

Ophthalmol. & Visual Science, 35:4328-4332; Toole, In Guidebook to the
Extracellular Matrix and Adlecien Proteins (eds. Kreis and Vale), pp. 64-65. (1993).

SUMMARY OF THE INVENTION

5   The present invention provides a method and a composition for modulating
wound healing in a tissue of interest in a mammalian host. The composition includes
a cell proliferation-modulating moiety joined to a targeting moiety that binds with
high affinity to a component of the extracellular matrix of the tissue of interest and/or
of the plasma membrane of a cell type that is involved in the wound-healing process.

10   The cell proliferation-modulating agent can be either one that inhibits or one that
promotes healing of the wound. The method includes the step of introducing the
composition into a host, generally in the area of the tissue of interest. The method
and compositions find use, for example, in the prevention or treatment of
proliferative vitreoretinopathy and in enhancing the success of glaucoma filtering

15   surgery by use of an antiproliferative agent as the cell proliferation-modulating agent
to inhibit growth of cells migrating into a wound site.

BRIEF DESCRIPTION OF THE DRAWINGS

         Figure 1 shows a schematic representation of one embodiment of the invention

20   wherein a wound in a tissue of interest is contacted with a composition which
includes a conjugate between a cell proliferation-modulating agent and a targeting
agent that binds with high affinity to a component of the ECM of the tissue of
interest. In this embodiment, the cell-proliferation modulating agent is daunomycin
attached to the carrier agent albumin, which is bound to the targeting-agent-which is a

25   monoclonal antibody specific for ECM components. The binding region of the
monoclonal antibody binds to a component of the ECM, thus placing the daunomycin
in close proximity to the offending fibroblast.

         Figure 2 shows a schematic representation of another embodiment of the
invention wherein the cell-proliferation modulating agent is a cytotoxic agent attached

30   to the targeting agent, which is an ECM-binding protein. The binding region of the
ECM-binding protein binds to a component of the ECM, thus placing the cytotoxic
agent in close proximity to the offending fibroblast.

Figure 3 shows a schematic representation of another embodiment of the invention wherein the cell-proliferation modulating agent is a cytotoxic agent, a ricin A chain, which is attached to the targeting agent, which is a bispecific antibody with specificity both for ECM components and for fibroblasts. The ECM-specific binding

5 region of the antibody binds to a component of the ECM, and the fibroblast-specific binding region of the antibody binds to fibroblast so that the cytotoxic agent can be internalized by the fibroblast and kill it.

BRIEF DESCRIPTION OF THE SPECIFIC EMBODIMENTS

10 Methods and compositions are provided for modulating healing of a wound site in a mammalian tissue of interest. The tissue can be any tissue which is characterized by having an extracellular matrix (ECM) composed of one or more components that can serve as a target for the targeting moiety of the conjugate, for example, hyaluronan and polypeptides such as proteoglycans, fibronectin, collagen,

15 certain glycoproteins and the like, and in which migration of particular cell types, such as fibroblasts, macrophages, platelets, polymorphonuclear cells and the like, into the wound site is part of the wound-healing or wound-closure process. Such cell types can also serve as targets. The compositions include a targeting moiety that "targets" a cell proliferation-modulating moiety to one or more components of the

20 ECM and/or cells that migrate into a wound site so that growth of cells migrating into the wound site is either increased or decreased, depending upon the nature of the cell-proliferation modulating moiety. Optionally, a linker agent can be included in the conjugate so that multiple molecules of the proliferation-modulating component can be associated with a single molecule of the targeting moiety. In some

25 embodiments, the targeting moiety of the conjugate is a bispecific agent with combined specificity for cell targets such as fibroblasts and phagocytes and components of the ECM in the tissue of interest.

The composition and method of the subject invention offer several advantages over currently available treatment modalities for glaucoma. Successful filtering

30 surgery can prevent many of the problems associated with medical therapy for POAG. These problems include patient compliance, ocular and systemic side effects associated with chronic anti-glaucoma therapy and pressure spikes. Current intra- and post-operative medical therapy employs anti-proliferative agents such as

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5-fluorouracil (5-FU) and mitomycin C that act nonselectively on all cell types within the anterior segment and ocular adnexa. Diffusion of these agents through tissue is essentially uncontrolled. Extensive diffusion at effective concentrations may be responsible for poor healing of surgical sites, and lead to complications. Limited

5   pharmacokinetic studies have been performed in this area, however, recent reports have suggested that drugs can diffuse through the sclera and be detected in the aqueous humor. Furthermore, 5-FU requires repeated injections (a typical treatment regimen is 1 to 2 injections per day for 14 days) and has a number of undesirable side-effects, which include epithelial and conjunctival ulceration, conjunctival wound

10   leaks, retinal toxicity, increased likelihood of ocular infection and pain during administration. With the subject invention, a single application is often sufficient for modulating wound healing following GFS. Furthermore, because the targeting moiety of the conjugate binds with high affinity to a component of the ECM, and/or a cell migrating into the wound site, the growth-modulating component of the conjugate

15   remains localized in the wound site, creating a high local concentration of the growth-modulating agent and minimizing diffusion of the growth-modulating agent into tissue abutting the wound site.

       Thus, the subject invention offers advantages which include controlled dosage of the growth-modulating agent and prevention of unwanted effects on essential cells

20   in the tissue of interest: localization of the conjugate to the wound site prevents migration to areas of sensitive cells, especially normal cells, from the wound site and sequesters the growth-modulating agent in the region of invading cells. Another advantage is that it is unnecessary to prepare targeting agents for each tissue of interest, as components of the ECM such as hyaluronan, collagen and fibronectin, are

25   common to many tissues. Specificity is instead achieved by the site of introduction of the conjugate. The resulting effect on the cell type can, as appropriate, include complete elimination of an undesirable cell type from the wound site.

       The wound-healing modulating composition is a conjugate comprising a cell proliferation-modulating agent and a targeting agent (first member of a specific

30   binding pair) that binds with high affinity to a component of the extracellular matrix (ECM) of a tissue of interest or at the plasma membrane of a cell involved in healing of the wound. The binding site on the ECM or the cell is the second member of the specific binding pair. By high affinity is intended higher affinity binding to a

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component of the extracellular matrix or cell surface as compared to other components of the ECM or other cells that may be present in the wound site or in tissue abutting the wound site. In general, where the targeting agent binds to a receptor on the extracellular matrix, the dissociation constant (K_d) is about 10^{-10}M to

5   10^{-6}M, preferably less than 10^{-8}M, most preferably less than 10^{-7}M. Where the targeting moiety is an antibody binding to an epitope on the extracellular matrix, the K_d will be on the order of about 10^{-8}M to 10^{-10}M, preferably less than 10^{-9}M. For other targeting agents, such as a peptide binding domain, such as the collagen-binding domain of fibronectin or the hyaluronic acid binding domains of a number of

10   proteins, i.e., link protein, aggrecan, hyaluronectin, the affinity of the binding domain for the relevant component of the ECM should be sufficient so as to provide specificity of binding to the ECM components.

The targeting agent can be a polyclonal or monoclonal antibody with high affinity for collagen, fibronectin, proteoglycans such as chondroitin sulfate,

15   glycoproteins unique to the extracellular matrix of the tissue of interest, or the like. Where the antibody is a monoclonal antibody, it may be produced as a result of hybridoma formation and expression by the hybridoma, or the supernatant culture, or produced by ascites, or may be a monoclonal antibody fragment, such as FAB, F(ab')$_2$, Fv, a recombinant variable region, or the like. The antibody may be of any

20   mammalian species, including, murine, rabbit, human or the like, or combinations thereof, such as chimeric antibodies, having a human constant region and a mouse or other mammalian source variable region. The important characteristic is that the antibody or antibodies bind with high affinity to one or more components of the extracellular matrix of the tissue of interest.

25   The methods for preparing such antibodies are well established. *See*, for example, Fazekas de St. Groth *et al.* (1980) *J. Immunol. Methods.* 35:1. An animal is hyperimmunized with a suitable immunogen, with or without addition of adjuvant. The source of the immunogen can include specimens such as tissues removed during surgery, biopsy specimens, cultured cell lines and the like. Various components of

30   the extracellular matrix of the tissue of interest or wound-healing cells which migrate into the wound site, such as scleral fibroblasts, macrophages, platelets, and the like may be used as the immunogen, particularly components that are obtainable from the human tissue of interest or human cells where the intended treatment recipient is

human, or from another intended recipient animal, where the intended use is a veterinary use or when a conjugate that is cross-reactive with human and animal tissue is desired. Thus, the immunogen can be extracellular matrix obtained from the tissue of interest, for example, human scleral tissue, human conjunctival tissue,

5   human vitreous and human epiretinal membrane. Alternatively, purified components obtainable from the extracellular matrix of the tissue of interest can be used as the immunogen, for example, collagen, fibronectin, proteoglycans, glycoproteins, and the like. Where a particular isotype of a protein is found in the tissue of interest as compared to other tissues, the isotype can be used as the immunogen, for example,

10 where the tissue of interest is human sclera, the immunogen can be type I or III human collagen, where the tissue of interest is vitreous, the immunogen can be type II human collagen.

In accordance with the subject invention, a mammal, which can be a mouse or other mammal, is hyperimmunized with immunogen according to methods well

15 known to those skilled in the art. Once a suitable antibody titer has been obtained in serum of immunized animals, antibody-producing cells such as spleen cells, lymph nodes or lymphocytes from an immunized animal are removed and immortalized using methods well known to those skilled in the art. Hybridomas are prepared and antibodies secreted by the hybridomas are screened to identify the clones that secrete

20 antibodies of the desired specificity. Screening of the hybridoma clones can be by binding to components of the extracellular matrix using an enzyme-linked immunoabsorbant assay (ELISA) or by other screening methods which are known to those skilled in the art, including radioimmunoassay and immunohistochemical staining of frozen sections of the tissue of interest. In addition to screening for

25 binding to components of a particular ECM type, the antibodies also were screened for species specificity and cell type specificity.

For some applications of the subject invention, it may be desirable to use bispecific antibodies rather than monospecific antibodies. By bispecific antibodies is intended antibodies which bind to epitopes on two different substrates, for example

30 they bind both to a component of the ECM and to a component in the plasma membrane of a particular cell type. The bispecific antibodies can be prepared in a number of ways, including linking or combining a monoclonal antibody specific for ECM components of the tissue of interest with another monoclonal antibody specific

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for a cellular target, such as a fibroblast cell or a retinal pigmented epithelial (RPE) cell. The two antibodies are joined using, for example, protein A or G. Mixing of different antibodies with protein A can lead either to precipitation or to an ill-defined product, however. In another method, parent hybridoma cell lines each producing an

5   antibody with a desired specificity are fused as described by Milstein and Cuello, (1984) *Nature* 5:299-304. This technique results in hybridoma cell lines which secrete antibodies of the types produced by each of the parent hybridoma cell lines. With multiple possibilities for heavy and light chain mixing, a heterogeneous population of antibodies can result. However, in some cases the predominant forms

10   are the parental antibodies and a bispecific form.

Fused hybridoma cell lines producing the desired bispecific antibodies can be obtained as described by Fazekas De St. Grogh and Scheidegger (1980) *J. Immunol Methods* 35: 21-1 and other methods known to those skilled in the art. The hybridoma producing the bispecific antibody can then be grown in large scale culture

15   and purified. Since the parental antibodies typically can have distinctly different isoelectric points, purification is achieved by cation exchange chromatography, for example, on Sepharose fast S (Pharmacia). Fractions from the chromatography can then be analyzed for specificity of binding and purified antibody which binds only to the cells of interest, components of the ECM in the tissue of interest, or both the cells

20   of interest and components of the ECM in the tissue of interest obtained. Examples of bispecific antibodies which have been obtained using this method include the bispecific antibody 1B4, a fusion of hybridomas 3D4 a number of normal epithelial cells including corneal and len epithelial as well as a number of epithelial derived tumor cell lines for example ME-180 cells and 7G12 (vitreous specific; binds to an

25   ECM component of the vitreous) and the bispecific antibody 7B5, a fusion of hybridomas 7H1 (cell specific, binds to a number of normal fibroblast cells derived from various animals for examle human and pig scleral fibroblasts, RPE cells and various cell lines i.e., ME-180 and MRC5) and 7G12 (vitreous specific). Of particular interest are components of the ECM in the vitreous and the sclera. Of

30   particular interest as cell types of interest are scleral fibroblasts, particularly human scleral fibroblasts and RPE cells.

Where the targeting agent is other than an antibody, it can be a protein which binds to extracellular matrix components and includes extracellular matrix binding

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proteins. Of interest are proteins that bind to hyaluronic acid (HA) (also referred to
as hyaluronan), and peptides derived from domains of these proteins which are
responsible for binding to HA. Several HA binding proteins have been described
including CD44, hyaluronectin/versican, aggrecan and link protein. These proteins

5   share a homologous region containing the HA binding domain. In addition, a novel
HA binding sequence unrelated to the above has been described by Hardwick, ((1992)
J. Cell Biol. 117:1343-1350). HA binding domains also have been described for
fibronectin and fibrinogen. These peptides can then be linked to drugs directly or by
the use of carriers. Equivalent peptides would those peptides which bind HA and that

10   show greater than 50% ssequence homology with known peptides binding HA, i.e.,
link protein.

        Peptides may be prepared using art accepted methods for solid phase
syntheses. Peptides prepared with a terminal cysetine or having a terminal control
group as described by Winger et al., Biocanjugate Chem. (1995) 6:323-326) can be

15   utilized for direct coupling to appropriately derivatized carrier proteins conjugated to
daunomycin. Carrier proteins are derivatized with thiol reactive groups such as
N-succinimidyl-32-pyridyldithio propinate (SPDP). Alternatively, peptides may be
synthesized such that they have a terminal hydrazide group. Such peptides also can
be prepared using conventional methods for solid phase synthesis. Once the final

20   amino acid has been added to the peptide, the terminal amino group is deblocked and
treated with succinic anhydride. Succinic anhydride reacts with the amino group to
provide a terminal carboxyl group to the peptide. Tert-butyl carbazate (Aldrich) is
reacted with this carboxyl group using carbodiimide. An alternative procedure is to
react eqimolar amounts of succinic anhydride with tert-butyl carbazate independently.

25   The product of this reaction is then reacted to the deblocked terminal amino group of
the peptide using carbodiimide. Final deblocking and decoupling yields the peptide
of interest with a terminal hydrazide group. The terminal hydrazide group can then
react with the C13 carbonyl group of doxorubicin or daunomycin forming a
hydrazone (Yamamoto, et al., (1972) J. Medicinal Chem. 15:872-875).

30   The choice of an agent which provides for modulation of wound healing will
depend upon whether inhibition or promotion of wound healing is the desired
outcome of treatment. For example, where the intended application is an inhibition
of wound healing, specific proliferation-modulating agents include daunomycin,

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mitomycin C, daunorubicin, doxorubicin, 5-FU, cytosine arabinoside, colchicine, cytochalasin B, bleomycin, vincristine, vinblastine, methotrexate or the like. Also of interest are toxic agents which may be derived from microorganism or plant sources. Examples include the toxic subunits of naturally occurring toxins such as ricin, abrin,

5 diphtheria toxin, saporin, and the like. Illustrative toxic subunits include the A chains of diphtheria toxin, enzymatically active proteolytic fragments from *Pseudomonas aeruginosa* exotoxin-A, ricin A-chain, abrin A-chain, modeccin A-chain, and proteins having similar activity found in various plants such as the plants *Gelonium multiflorum, Phytolacca Americana, Croton, Tiglium, Jatropha, Curcas, Momordic,*

10 *Charantia, Reachan,* the toxin saporin from *Saponaria officinalis* (Thorpe *et al.*, (1985) *J. National Cancer Institute* 75:151), the Chinese cucumber toxin, trichosanthin (Yeung *et al.*, (1985) *Intl. J. of Peptide Protein Res.* 27:325-333). Mutant species of the toxins also may be used, for example, CRM 45 (Boquet *et al. Proc. Natl. Acad. Sci. USA* (1976) 73:4449-4453). Where the desired result is

15 enhanced wound healing, the proliferation-modulating agent can be a growth-promoting agent such as fibroblast growth factor, epidermal growth factor, transforming growth factor-β, and platelet-derived growth factor.

The proliferation-modulating agent and the targeting agent providing for binding to the extracellular matrix can be linked, usually by a bond which is

20 cleavable, either intra- or extracellularly by reduction, hydrolysis, enzymatically, or by a bond which is acid labile. The type of linkage used depends upon a number of factors, but particularly the nature of the proliferation-modulating agent. For example, where the agent is one which must be internalized by a cell to have an effect, such as a toxin molecule or toxin A chain, it is preferable that the linkage to

25 the targeting moiety can be cleaved. The targeting moiety can be linked to a drug either directly or indirectly by carrier molecules such as serum albumin (particularly human serum albumin), polyaminoacids, dextran, and the like, by methods well known to those skilled in the art. The use of a carrier molecule permits binding of multiple molecules of the proliferation-modulating agent to the linker molecule, the

30 number at least partially dependant upon the size of the carrier. Generally, 10 to 30 molecules of agent are bound per molecule of carrier molecule, preferably 5-20, for an antiproliferative compound, and 1 to 2 molecules per molecule of carrier molecule for a toxin molecule.

The type of linkage used can also be dictated by the cell type which is the ultimate goal of the proliferation modulating activity thus preferably the linkage is a pH labile or acid labile link between the targeting moiety and the proliferation-modulating moiety in cases where the cells have phagocytotic properties which

5   migrate into the wound site, for example, fibroblasts and macrophages. Also of interest are peptide linkages which are susceptible to hydrolysis by enzymes which are present in the extracellular matrix. Convenient linkages thus include disulfides, imides, hydrazones, amides and the like.

For some applications it is desirable that the wound-modulating conjugates be

10   associated noncovalently or covalently in an amount sufficient to provide the desired amount of cell with a compound such as hydroypropylemethyl cellulose (HPMC), collagen, destrem, etc. modulation, either cell-proliferation inhibition or enhancement. The conjugate can be incorporated into wafers (or other forms) comprising materials such as HPMC, collagen, dextran, etc. The conjugate would

15   not necessarily be bound to the mixture rather than incorporated with these materials. This is intended to cement and aid the delivery of the conjugate. A wafer could be delivered to a surgical site more efficiently than an injection.

The conjugate can be associated nonconvalently with the polymer either by entrapment of the agent within the biologically inert polymer or by adsorption on to

20   the biologically inert polymer. Generally the rate of release of the growth-modulating agent from the will be at the rate of about 25 to 50% of the amount bound per hour. Generally, the growth-modulating agent is combined with the solid support by assembly of the various components in a sterile environment and the assembly is maintained in an aseptic environment until use.

25   Other agents also can be employed in conjunction with the process of this invention. For example, all-trans retinoic acid can be used to enhance the receptor-mediated cytotoxicity of ricin A-chain immunotoxins, and anti-inflammatory agents can be used.

Corticosteroids	Name	Trade Name
	Dexamethazone	Maxidex, Decadron, AK-Dex
	Fluorometholone	Fluor-Op, FML, Flarex
	Prednisolone	Pred-Forte, AK-Pred, Inflamase
Nonsteroidal		
	Diclofenac	Voltaren
	Flurbiprofen	Ocufen
	Ketorolac	Acular

The effectiveness of the conjugate for its intended use may be determined in a variety of ways. For example, where the desired effect is inhibition of cell proliferation, particularly proliferation of cells which migrate into an ocular wound, it is preferable to test the conjugate using cells as similar as possible to the cell type in the tissue of interest, since the sensitivity of various cell types to growth-modulating agents may vary significantly. For example, where the intended use of the conjugate is in human ocular tissue, it is preferable that human scleral fibroblast cells obtained from surgical specimens or fresh eye bank tissues are used; conversely, where the intended use is a veterinary one, it is preferable that the cells be from the intended recipient host species.

Conjugate effectiveness can be evaluated in any of several bioassays including *in vitro* cell culture of scleral fibroblasts and an *in vivo* model of the intended condition for treatment, such as glaucoma or PVR. As an example of an *in vitro* assay, for determining the ability of the conjugate to inhibit or stimulate cell proliferation *in vitro*, cell culture plates can be coated with an extracellular matrix component of the type most prevalent in the tissue of interest. As an example, type I or type III collagen is prevalent in scleral tissue, type II collagen is prevalent in vitreous. Various concentrations of a conjugate having a high affinity for the ECM component used to coat the plates is then added. The plates are incubated to allow binding of the conjugate to the well associated ECM component, then washed to remove any unbound conjugate. The plates are then washed to remove any unbound

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conjugate. Culture plates are then seeded with a sub-confluent concentration of cells from the tissue of interest, for example, scleral or conjunctival-derived fibroblasts or RPE cells, and incubated for a sufficient time to observe the desired effect. Growth is determined for each plate by any method known to those skilled in the art, such as

5   total cell count used to differentiate between live and dead cells, trypan blue dye exclusion, tritiated thymidine incorporation and the like. An effective concentration is defined as the dose which inhibits fibroblast proliferation by at least 70%, preferably more than 80%, and most preferably by more than 95% when compared to control plates, i.e., those to which targeting moiety alone is added, or as the dose that

10   stimulates cell proliferation by at least 50%, preferably more than 100% percent, and most preferably by more than 200% when compared to control plates over a period of 24-48 hours.

The conjugates of the subject invention have several uses. In one application of the invention, the tissue(s) of interest are the sclera and/or the conjunctiva of the

15   eye, and the intended application of the invention is to enhance the success rate of glaucoma filtering surgery, in which a drainage fistula or channel has been surgically created to increase aqueous humor outflow as a means of lowering IOP. The intended application of the subject invention in glaucoma filtering surgery is to control healing of the surgical wound such that it heals, but in the process does not create excessive

20   scar tissue to block the filtering channel and bleb that have been made. Since many of the cells in the tissue abutting the wound site are amitotic (non-dividing), the antiproliferative agent will have no effect on these cells, thus conferring additional selectivity to the use of the conjugate.

For evaluation of the efficacy of the invention for use as an adjunct in

25   glaucoma filtering surgery, an *in vivo* animal model is used. A functioning filter is created in ocular tissue of rabbits according to the methods described by Lee, *et al.*, (1987) *Ophthalmol.* 94:1523. Pigs, monkeys, dogs, or other animals that provide an art-accepted means for testing glaucoma treatment regimens also can be used as the model animal. Various concentrations of the immunoconjugate are administered to

30   the fistula and bleb areas prior to, during, or following surgery. Eyes are then studied in terms of IOP, outflow facility and percent of eyes with functional filters as determined by ocular examination. At selected times following surgery, animals are humanely sacrificed and the eyes are submitted for histologic evaluation to assess

15

ultrastructural changes in the fistula and filtering bleb. Efficacy of the immunoconjugate is determined by a reduction in IOP, increase in outflow facility and increase in mean duration of time that functional filters are observed when compared to vehicle-treated (control) animals.

5   Another use of the invention is in the prevention of proliferative vitreoretinopathy (PVR). In this disease, cells migrate into the posterior chamber of the eye following accidental or surgical trauma. The clinical presentation will vary depending on whether the condition originated from pre-existing retinal tears (rhegmatogenous retinal detachment) or as a result of other ocular diseases such as

10   diabetes, retinopathy of prematurity or from injury. Retinal detachment has been observed following intracapsular surgery and Nd:YAG laser capsulotomy. Various cell types implicated in the formation of PVR include: retinal pigmented epithelium (RPE), glial cells, fibroblasts, macrophages and blood elements. The usual presentation of PVR is characteristic of the elements of wound healing. Cell type,

15   presence of various growth and differentiation factors, as well as the generation of various extracellular matrix components (ECM) combine to promote the inappropriate formation of contractile membranes.

Extracellular matrix components in epiretinal membranes have been analyzed and show collagen and fibronectin to be the major constituents. The preferred animal

20   model for PVR, which can be used to test the efficacy of the conjugate in treating PVR in rabbits, involves compression and partial detachment of the vitreous humor from the retinal surface, followed by introduction of fibroblasts into the posterior chamber of small numbers of cultured fibroblasts (Chandler, et al., (1986) Arch. Clin. Exp. Ophthalmol. 224:86-91). The bispecific or a monospecific conjugate can

25   be used. The experimental animal, for example, a rabbit, is anesthetized and the pupils of the eyes dilated. Cryopexy is performed and seven days following cryopexy, a gas such as perfluorocarbone is injected intravitreally under indirect ophthalmoscopic observation. The gas is left in the eye for about two days after which it is replaced with a balanced salt solution. Seven to ten days after gas-fluid

30   exchange, the eyes are examined for indications of cataract, vitreous hemorrhage, or any retinal detachment. Animals with any sign of these conditions are eliminated from the study. Experimental eyes are injected with cells such as fibroblasts directly above the optic disc. The test conjugate is injected into the posterior chamber of the

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eye both before, at the time of, or following injection of the fibroblasts. Following injection, animals are monitored for adverse reactions and toxicity using ophthalmoscopic and slit lamp examination of the cornea, anterior chamber, iris and retina Fundus photographs or drawings are obtained at various intervals following

5   fibroblast injection. PVR development is graded using the classification system of Hida, *et al.*, (1987) 225:303-307 *Arch. Clin. Exp. Ophthalmol.* Eyes are removed from the animals and processed for histological examination and/or immunocytochemical staining to analyze binding of the targeting agent to epiretinal membranes. This model has been used to study daunorubicin (Khawly, *et al.*, (1991)

10   *Arch Clin Exp, Ophthalmol*, 229: 464-467; Santana, *et al.*, (1984) *Arch Clin Exp, Ophthalmol*, 221: 210-213; aclacinomycin (Steinhorst (1994) *Invest Ophthal Vis Sci*, 35(4) 432 and retinoic acid in silicone oil (Araiz, *et al.* (1993) *Invest. Ophthal. Vis. Sci.* 34:522-530) as treatments of PVR.

In using the subject invention for treatment, a sufficient amount of the growth

15   modulating agent is introduced into the wound site to achieve the desired effect of enhanced wound healing or inhibition of wound healing. Methods for introducing the growth-modulating agent include injection directly into the wound site of a solution comprising the cell-modulating agent. An effective dose of the conjugates for inhibiting wound healing in glaucoma filtering surgery would generally be in the

20   range of 1-500 ug/0.1 ml vehicle, preferably 1-100 ug/0.1 ml vehicle, and most preferably 1-50 ug/0.1 ml vehicle. For treating PVR, an effective dose of the conjugates would generally be in the range of 1-50 ug/0.1 ml vehicle, preferably 1-25 ug/ml vehicle, and most preferably 1-10 ug/0.1 ml vehicle. The vehicle may be any pharmaceutically acceptable solution that is compatible with the conjugates, including

25   phosphate-buffered saline, balanced salt solution and the like. Compatible pharmaceutical stabilizating agents, antiseptic agents and the like may also be used in the conjugate solutions as long as they do not adversely interfere with the activity of the wound-healing-modulating conjugates.

Where the growth modulating agent is used in conjunction with a surgical

30   procedure, it can be introduced before, during or after surgery, preferably at the time of surgery into the wound site. The wound-healing-modulating conjugate may be introduced during the surgery incorporated in a solid support composed of materials including biodegradable polyanhydrides and other biocompatible polymers such as

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HPMC, dextran or collagen. The wound-healing-modulating agent also can be applied after the surgical incision in the conjunctiva, but before an incision is made in the sclera, to allow the agent to bind to the newly exposed target tissue. Alternatively, it may also be applied 1 to 3 days after surgery by injection at or near

5   the surgical site.

Generally, the affect of the cell-modulating agent associated with the ECM on cell proliferation will be realized within 3 to 24 hours after cells come in contact with the cell-modulating agent, depending upon the concentration of the agent used and/or the mode of application.

10   In some applications, such as scarring of a fistula site in GFS, it may take from 7 to 30 days. Blockage of the drainage fistula is accompanied by a concomitant rise in IOP and loss of bleb patency. These clinical signs of the failure of the fistula and/or the filtering bleb are evaluated by pneumotonometry or other methods and by visual inspection and digital examination of the bleb site.

15   The subject compositions can be provided as kits for use in one or more operations. Kits will include the growth-modulating conjugate either as a concentrate, including lyophilized compositions (which may be further diluted prior to use or at the concentration of use, where the vials may include one or more dosages. Conveniently, single dosages may be provided in sterilized containers. Where the

20   containers include the formulation for direct use, usually there will be no need for further reagents for use with the method.

The following examples are offered by way of illustration and not by way of limitation.

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Biological Materials Deposits

        The following hybridomas, which secrete monoclonal antibodies described
below, were deposited on June 8, 1994, with the ATCC under the indicated accession
numbers:

Hybridoma	ATCC Accession Number
7G12	_____
6G10	_____
7H1	_____
1B4	_____
7B5	_____

20

<u>Example 1</u>

<u>Preparation of Goat Anti-Collagen/Daunomycin</u>

<u>Immunoconjugates</u>

a.   <u>Preparation of Goat Anti-Collagen Antibodies</u>.

5   A castrated male Spanish goat, 3-5 years of age, was immunized with type I

human collagen (Calbiochem) emulsified in Freund's complete adjuvant (Difco). The

animal was given 50 ug collagen in adjuvant as 6-8 subcutaneous injections

(prescapular, post-scapular and lateral lumbar regions) at 0.1 - 0.2 ml per site with a

total injection volume of 1.0 ml. Booster injections were given every two weeks for

10   six weeks. Antibodies to type I collagen were detected by ELISA, the animals were

bled and serum obtained by conventional methods.

b.   <u>Preparation of Affinity-Purified Anti-Collagen Antibodies</u>.

A purified immunoglobulin (IgG) fraction was prepared from immune goat

serum by ion exchange chromatography. Serum (360 ml) was diluted ten fold in 50

15   Mm MES buffer, pH 6.0 and clarified by passage over approximately 800 ml of Cell

Debris Remover (CDR) (Whatman) using a Buchner funnel. The diluted and clarified

serum was applied to a Fast S (Pharmacia) cation exchange column (100 ml bed

volume) equibrated in the same buffer used to dilute the serum. The bound IgG

fraction was eluted with 50 Mm MES pH 6.0 containing 200 Mm NaCl and dialyzed

20   overnight against phosphate-buffered saline (PBS) at 4°C. The purified

immunoglobulin fraction was applied to a 20 ml gelatin-Sepharose 4B column

(Pharmacia). Bound antibody was eluted with 200 Mm glycine-Hcl buffer pH 2.5.

Affinity purified antibody (40 ml) was dialyzed overnight against 50 Mm MES buffer

pH 6.0 and passed over a 1.5 ml Fast S column equilibrated in the same buffer.

25   Bound antibody was eluted with 50 Mm MES pH 6.0 containing 200 Mm NaCl. The

antibody solution was desalted into PBS using a Econo Pac 10 DG column (Bio-Rad).

Protein concentration was determined by absorbance at 280 nm. Six ml of affinity-

purified antibody at 1.3 mg/ml was obtained.

c.   <u>Preparation of Anti-Collagen Daunomycin Immunoconjugates</u>.

30   Affinity-purified goat anti-type I collagen is conjugated to daunomycin by a

method modified from Yang <i>et al</i>. (1988) <i>Proc. Natl. Acad. Sci.</i>, <u>85</u>:1189-1193.

Briefly, 5 mg of cis-aconitic anhydride (Sigma) is dissolved in 0.050 ml dioxane and

added dropwise to an ice-cold solution of daunomycin (DM) (10 mg in 1.0 ml 400

Mm sodium bicarbonate buffer ph 9.0). The pH is maintained at 9.0 with the
addition of 0.5N NaOH. After 15 min., the pH is adjusted to 7 and 0.4 ml of 1 M
Hepes buffer added. After incubation for one hour at room temperature, six mg of 1-
ethyl-3-(3-dimethylaminopropyl-carbodiimide (Sigma) and six mg N-

5   hydroxysulfosuccinimide (Pierce) is added to the reaction mixture. Six mg of affinity
purified antibody (10 mg/ml) in 50 Mm Hepes buffer pH 7.0 is added and the
reaction allowed to proceed overnight at room temperature. The immunoconjugate is
purified by passage over a Econo Pac 10 DG desalting column equilibrated with PBS.
The concentration of DM is determined by measuring the absorbance at 476 nm

10   (extinction coefficient of 17.3 at 1mg/ml). The IgG concentration is determined
using the Bradford Coommassie Blue dye binding assay (Bio-Rad).

d.   _In vitro_ Inhibition of Cell Growth with Anti-Collagen Daunomycin
        Immunoconjugates.

The anti-type I collagen-DM conjugate cytotoxicity can be tested on human or

15   rabbit scleral derived fibroblasts. Briefly, 24 well culture plates are precoated with
rat tail collagen (Collagen Corp.) 0.2 ml/well, 200 ug/ml and the collagen allowed to
dry overnight at room temperature. The following day additional wells are loaded
with 1.0 ml PBS containing 0.1% BSA and the plates are incubated for an additional
2 hours at 37°C. Wells are washed twice with PBS to remove unbound protein.

20   Dilutions of immunoconjugate in PBS containing 0.1% bovine serum albumin (BSA)
are prepared and added to wells. PBS containing 0.1% BSA was added to collagen-
and BSA-treated wells as controls. The plates are incubated at 37° for 3 hours to
allow for immunoconjugate binding, then washed twice with PBS to remove unbound
material. A suspension of human scleral fibroblasts in M199 media (Gibco)

25   containing 10% fetal bovine serum (FBS) is added to each well (1.0 ml/well at 1 X
10^4 cells/ml). Plates are incubated at 37° for 48 hours followed by the addition of
2.0 uCi 3H-thymidine (Amersham) and incubation for 24 hours. Following
thymidine incorporation, cell monolayers are rinsed three times with PBS and 1.0 ml
5% ice cold trichloroacetic acid (TCA) added to the wells. After 30 minutes on ice

30   the well are rinsed once with 5% TCA and the precipitate dissolved by the addition
of 1.0 ml 0.1 N NaOH containing 2% Na_2CO_3 and incubation at 37°C for one hour.
Following neutralization of the samples by the addition of 15 ul 12 N Hcl/well, the

amount of radioactivity in each well is determined by adding 0.5 ml of each sample
to 9.0 ml scintillation fluid and counting in a Beckman scintillation counter.

Example 2
Preparation of Murine Monoclonal Antibodies to
Extracellular Matrix Components

Preparation of hybridomas secreting monoclonal antibodies to extracellular
matrix components follows standard procedures developed in a number of laboratories
(Fazekas de St. Groth *et al.* (1980) *J. Immunol. Methods*, 35:1-21; and Goding,
Monoclonal Antibodies: Principles and Practice, Academic Press, London, 1986).
Individual female BALB/c mice, (4 to 6 weeks of age) were immunized with human
scleral tissue (homogenized in PBS), bovine plasma fibronectin (pFN) (purified from
bovine plasma by gelatin-sepharose affinity chromatography) and human vitreous.
Antigen preparations were emulsified in Freunds complete adjuvant (Difco). Mice
were given 50-100 ug of antigen preparation injected subcutaneously. Booster
injections were prepared in Freunds incomplete adjuvant. Three days prior to fusion
mice were given a final boost of antigen in PBS given as an intraperitoneal injection.

One day prior to cell fusion, peritoneal macrophage feeder cells were
prepared. Five BALB/c mice were sacrificed by CO_2 asphyxiation and their
peritoneal cavities flushed with 5 ml sucrose solution (0.34M). Washings were
pooled, counted and centrifuged. Cells were resuspended in Iscoves-modified
Dulbeccos containing hypoxanthine, aminopterin and thymidine (IMDM) media to a
concentration of 6×10^4 cells/ml. The cells were then distributed in ten 96 well
plates at 100 ul/well and incubated at 37°C in a CO_2 incubator.

The P3-X63/AG8.653 myeloma cell line (ATCC # CRL 1580) was grown in
IMDM containing 10% FBS. The cells were maintained in log phase growth at low
density and split 1:2 the day before the fusion. On the day of fusion myeloma, cells
were collected and washed in IMDM media with 10% serum. The final wash and
resuspension was in IMDM without serum. Cells were suspended to a concentration
of 1×10^7 cell/ml. All procedures were performed at room temperature.

The spleen from each immune mouse was removed aseptically and minced to
a single cell suspension. Cells were suspended in 10 ml IMDM and large clumps
allowed to settle for 10 minutes. Nine ml of cell suspension were transferred to a 50

ml centrifuge tube, made up to 50 ml with IMDM and cells counted with hemocytometer. Cells were then centrifuged at 200 X g for 10 min and suspended at 1 X 10⁷ cells/ml in IMDM. The preparation of myeloma and spleen cells was carried out simultaneously.

5   For fusion, 10 ml of myeloma and 6 ml of spleen cells were combined with 34 ml IMDM and centrifuged at 200 X g for 10 min. The supernatant was removed and the pellet loosened by gentle agitation. One ml of 45% PEG 4000 (Gibco) was added dropwise over one minute with constant agitation of the tube. The tube was then immersed for 90 seconds in a 37°C water bath with gentle agitation and finally
10   centrifuged at 20 X g for 1 min. Fusion was stopped by slowly diluting PEG with IMDM. Twenty ml IMDM was added, 1 ml over the first 30 seconds, 3 ml over the next 30 seconds, and the rest over the second minute. The cells were then allowed to stand for 5 minutes, centrifuged for 10 minutes at 200 X g, and the supernatant discarded.

15   Following fusion, cells were suspended in IMDM media containing hypoxanthine, aminopterin and thymidine (HAT) and 20% FBS and plated on the previously prepared macrophage feeder cells to give approximately 1 X 10⁴ cell/well. Cells were refed on days 5, 12 and 15, with half (100 μl) of the media removed and replaced. When the positive cultures were identified by screening assays (see below),
20   they were transferred to 24 well plates containing 1 ml of IMDM media containing hypoxanthine and thymidine and 10% FBS. When confluent, the expanded cultures also were retested and positive cultures cloned, frozen and expanded. The expanded cultures were also tested and frozen. Further expansion and cloning was performed in media without hypoxanthine, aminopterin or thymidine.

25   Culture supernatants were tested for specific monoclonal antibodies by ELISA using antigen coated plates. Results of hybridomas selected from each fusion are shown in Table 1.

Table 1

Binding of Hybridoma Culture Supernatants

to ECM Antigens by ELISA[1]

Supernatant	Vitreous[2]	pFN[3]	Scleral Extract
7G12	0.75	0.08	1.2
3A3	0.10	1.2	0.13
6G10	0.01	0.02	1.0

[1]ELISA assays were performed in 96-well polystyrene test plates. Antibodies were detected using goat anti-mouse IgG coupled to peroxidase (Kirkegaard and Perry). ABTS was used as the substrate. Absorbance readings were determined with a Dynatech plate reader at 410 nm. [2]Rabbit vitreous diluted in PBS and dried overnight at room temperature. [3]Bovine plasma fibronectin diluted in PBS and incubated overnight at 4°C. [4]Human sclera tissue extracted in PBS using a Dounce homogenizer and dried overnight at room temperature.

Supernatants from hybridomas 7G12 and 6G10, that tested positive for human scleral tissue, were also examined for antibody binding to human type I collagen. Results are shown in Table 2.

Table 2

Binding of Hybridoma Culture Supernatants

to Human Type I Collagen by ELISA[1]

Supernatant	Type I Collagen	BSA
7G12	0.16	0.03
6G10	0.40	0.00

[1]The assay was performed in a 96-well polystyrene plate. Human type I collagen (Sigma) diluted in M199, added to wells and dried. Additional wells were treated with BSA diluted in PBS. Antibodies were detected with goat anti-mouse IgG coupled to peroxidase. ABTS was used as the substrate. Results are expressed as absorbance at 410 nm and are the average of duplicate samples.

Production of the Ascites fluid containing monoclonal antibodies and high concentration was performed as described by Harlow and Lane (Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratories (1988) Cold Spring Harbor, New York). Hybridomas cells were cultivated in cell culture media, harvested by

centrifugation and washed PBS. Cells (1-5 X 10⁶) were injected
intraperitoneally into adult females BOBA/C mice. Mice had been
primed by injection of 0.2ml 2,6,10,14-tertramethyl decanoic acid
(Pristane, Sigma) ten to 14 days prior to injection of cells. Ascities

5   fluid was harvested from the mice within three weeks following
injection of cells. The Ascities food was centrifuged at 3,000 x g
for ten minutes, cooled and stored at -20°C.

10   Example 3
                          Preparation of Murine Monoclonal
                            Antibodies to Ocular Tissues
        a.   Preparation of Antibodies Binding Scleral Fibroblasts and Retinal
               Pigmented Epithelial Cells.

15   Preparation of hybridomas secreting monoclonal antibodies to scleral
fibroblasts and retinal pigmented epithelial cells followed standard procedures
developed in a number of laboratories (Fazekas de St. Groth (1980); and Goding
(1986). Individual BALB/c mice were given interperitoneally human scleral
fibroblasts in PBS. Subsequent injections were given intravenously. Three days

20   prior to fusion, mice were given a final boost of cells by an intravenous injection.
Fusion procedures using P3-X63/AG8.653 myeloma were performed as in
Example 2. Culture supernatants were tested for antibodies binding human scleral
fibroblasts by ELISA. Positive cultures were expanded, cloned by limiting
dilution and frozen in liquid nitrogen. Results of binding assay using culture

25   supernatant from one positive hybridoma (7H1) to various cell types are given in
Table 3. Results of binding using culture supernatant from hybridoma 3D4 (US
Patent No. 5,055,291) is also given.
        b.   Preparation of hybridomas secreting bispecific antibodies 1B4 and 7B5.
               Hybridomas producing bispecific antibodies were produced as described by

30   Wong et al. (1987) J. Immunol. 139:1369-1374. This procedure results in
hybridoma cell lines secreting both types of antibody. With multiple possibilities

Table 3

Binding of Monoclonal Antibodies to Vitreous and Cells by ELISA[1]

	Vitreous[2]	ME180[3]	HSF[4]	RSF[5]	PSF[6]	RPE[7]	MRC5[8]
Parental							
7H1	0.01	0.77	0.32	0.08	0.30	0.59	0.87
3D4	0.01	0.75	0.02	0.22	0.04	0.13	0.02
7G12	0.54	0.02	0.02	0.08	NT	0.25	0.12
Bispecific							
1B4	0.86	0.21	NT				
7B5	0.64	0.14	0.52				

[1]ELISA assays were performed in 96-well polystyrene test plates. Individual cell suspensions were added to wells in M199 containing 10% FBS and allowed to attach by overnight incubation at 37°C. Sufficient cells were added to give complete confluence at the time of assay. Bound monoclonal antibodies were detected using goat anti-mouse IgG coupled to peroxidase (Kirkegaard and Perry). ABTS was used as the substrate. Absorbance readings were determined with a Dynatech plate reader at 410 nm.
[2]Pig vitreous diluted in PBS and dried overnight at room temperature.
[3]ME180, human cervical carcinoma cell line ATCC Number HTB33.
[4]HSF=human scleral fibroblast
[5]RSF=rabbit scleral fibroblast
[6]PSF=pig scleral fibroblast
[7]RPB=pig retinal pigmented epithelial cells
[8]MRC5, human fibroblast cell line ATCC Number CCL171.
NT=Not Tested

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for heavy and light chain mixing a heterogeneous population of antibodies can result. However, in some cases the predominant forms are parental antibodies and a bispecific antibody (Milstein *et al.* (1984) Nature, 305:537-540). 7G12 cells secreting a monoclonal antibody binding vitreous were made thymidine kinase

5   deficient (TK-) by incubation in IMDM containing 10% FBS and an increasing concentration of 5-bromo-2'-deoxyuridine (1.95 x 10-5 to 1.95 x 10-4 M). 3D4 and 7H1 cells (secreting antibodies binding cells as described above) were made hypoxanthine guanine phosphoribosyl transferase deficient (HGPRT-) by incubation in culture medium containing 8-azaguanine (3.3 x 10-5 M). Following

10   incubation in respective media surviving cells were subcloned by limiting dilution (0.5 cells/well) in 96 well culture plates and the subclones tested for viability in medium containing hypoxanthine, aminopterin and thymidine (HAT). Subclones sensitive to HAT and producing antibody were selected for fusion. TK- 7G12 cells were fused with HGRPT- 3D4 and 7H1 cells as described in Example 2.

15   Culture supernatants were tested for binding to both vitreous and fibroblasts or ME180 cells using procedures described above. A hybridoma culture testing positive for antibody binding to vitreous and ME180 cells (7G12X3D4 fusion) was labeled as 1B4. A hybridoma culture testing positive for binding to vitreous and fibroblasts (7G12X7H1 fusion) was labeled as 7B5. Results of ELISA assays for

20   parental and bispecific antibodies to their respective antigens is shown above in Table 3. Binding by parental 7G12 supernatant from Example 2 is also shown.

Example 4
Preparation and Assay of Bispecific Immunotoxins

25   a.   Purification of Bispecific Antibody 1B4.

1B4 hybridoma cells, prepared as described in Example 3, were grown in IMEM containing 10% FBS in spinner culture. Three liters of culture supernatant were obtained and clarified by passage over CDR, concentrated to 500 ml by ultrafiltration, diluted 1:10 with 50 Mm MES buffer pH 5.6 and applied to a

30   Fast S cation exchange column. 1B4 antibody was batch eluted with MES buffer containing NaCl. The column was first treated with buffer containing 125 Mm NaCl. Parental 3D4 antibody eluted at this concentration. 1B4 was then eluted with MES buffer containing 175 Mm NaCl. Parental 7G12 antibody was eluted

with 250 Mm NaCl in MES buffer. The eluted bispecific antibody was further

purified over protein A-Sepharose (Pharmacia) and the eluted antibody desalted

over an Econo Pac 10 DG column equilibrated with PBS.

5   b.   Conjugation of Ricin A with 1B4.

Purified 1B4 antibody was treated with a 7 fold molar excess of SPDP (1

mg/ml in ethanol) incubated for 1 hour at room temperature and desalted over a

Econo Pac 10 DG column equilibrated with PBS. SPDP derivitized 1B4 was

mixed with ricin A and incubated for 2 hours at room temperature followed by

10   4°C overnight.

c.   Cytotoxic Activity of Bispecific Immunotoxin 1B4-Ricin A.

Cytotoxic activity of 1B4-RA was tested essentially as described in

Example 1d. Briefly, 24-well culture plates were precoated with rabbit vitreous

15   diluted in PBS (0.2 ml/well, 6.2 ug/ml of protein) and dried overnight at room

temperature. The following day wells were fixed by the addition of absolute

ethanol for 30 minutes at 4°C and rinsed with M199 containing 10% FBS.

Additional wells were loaded with 1.0 ml PBS containing 1.0 % BSA and the

plates were incubated for an additional hour at 37°C. Wells were washed twice

20   with M199 containing 10% FBS. Dilutions of 1B4-RA in M199 containing 10%

FBS were prepared (250 ul/well at 250 ug/ml) and added to triplicate wells.

M199 containing 10% FBS was added to triplicate wells as a control and the plate

incubated at room temperature for 2 hours to allow for immunotoxin binding, then

washed three times with PBS to remove unbound material. A suspension of

25   ME180 cells in M199 containing 10% FBS was added to each well (2 X 10^4 cells

in 1.0 ml/well). Plates were incubated at 37°C for 48 hours followed by the

addition of 2 Uci per well 3H-leucine (Amersham) and incubation for 24 hours.

Following leucine incorporation, cell monolayers were processed as in Example 1d

and the amount of radioactivity in each well was determined by counting in a

30   scintillation counter. Results are show in Table 4. Figure 3 shows a schematic

representation of the bispecific immunotoxin.

Table 4

Cytotoxic Activity of Culture Wells Coated with

Vitreous and Treated with 1B4-RA for ME180 Cells

	3H Leucine Incorporation (cpm[1])	
Culture wells coated with:	Vitreous	BSA
Wells treated with:		
Media (control)	15,511	12,262
1B4-RA	3,272	18,211

[1]Individual culture wells were coated with a solution of rabbit vitreous diluted in PBS and then treated with 1B4-RA. Following incubation to allow for immunotoxin binding, the wells were rinsed with PBS to remove unbound conjugate and ME180 cells in M199 containing 10% FBS were added. Following incubation, the ability of cells to incorporate 3H-leucine was determined.

Example 5

Preparation and Assay of 3A3-BSA-DM Immunoconjugates

a.   Modification of Daunomycin using Cis-Aconitic Anhydride.

Thirty mg of daunomycin (Sigma Chemical Co.. St. Louis MO) was dissolved in 1.8 ml of ice cold water. Bicarbonate buffer (1.2 ml of a 1M solution, pH 9.0) was added and the solution was placed on ice with stirring. A solution of 0.15 ml dioxane containing 15 mg of cis-aconitic anhydride was added dropwise. The pH was maintained at 9.0 by the addition of 0.5 N NaOH. The reaction mixture was incubated for 15 minutes at 4°C with stirring. Following incubation, 1.2 ml of 1.0 M Hepes buffer, pH 7.0 was added and the reaction mixture was removed from the ice bath and incubated for 60 minutes. Following incubation 155 mg of ethyl-3-(3-dimethylaminopropyl)carbodiimide-Hcl (Pierce) and 150 mg N-hydroxysulfosuccinimide (Pierce) was added to the daunomycin solution and dissolved. The solution was allowed to incubate for 10 minutes and the final volume adjusted to 4.8 ml by the addition of 0.3 ml of 1 M Hepes buffer.

b.   Attachment of Modified Daunomycin to Albumin.

Bovine serum albumin (Sigma Chemical Company) was dissolved in 0.1 M MES buffer, pH 6.0. Twenty mg BSA in 1.12 ml was added to 3.2 ml of the daunomycin solution prepared above with stirring. The reaction vial was incubated overnight at room temperature in the dark to give BSA-DM.

30

c.   Introduction of Free Sulfhydryl Groups on BSA-DM.

Following overnight incubation BSA-DM was separated from free DM by
desalting over a Econo Pac 10 DG column equilibrated with 50 Mm MES buffer
pH 6.0. Limited reduction of disulfide bonds in the BSA-DM was performed by

5   adding dithiothretiol (DTT) to a final concentration of 50 Mm followed by
incubation at room temperature for 30 minutes. DTT was removed by desalting
over a Econo Pac 10 DG column equilibrated with PBS containing 1 Mm EDTA.
As a final step the BSA-DM solution was concentrated to a final volume of 1.1 ml
by ultrafiltration (molecular weight cutoff of 10,000 daltons) using a Centricon 10

10   (Amicon). A ratio of 22:1 DM molecules per BSA molecule was obtained.

d.   Modification of Antibody 3A3 with SPDP.

Purified 3A3 antibody (0.83 mg/ml in PBS) which binds fibronectin was
treated with a 7.5 molar excess of long chain (LC)-SPDP (Pierce) (1mg/ml (LC)-
SPDP in ethanol) and incubated at room temperature for 1 hour.

15   e.   Conjugation of 3A3 to BSA-DM.

3A3-(LC)-SPDP (0.9 ml) was added to 0.250 BSA-DM prepared from
Example 5c and incubated for one hour at room temperature followed by overnight
incubation at 4°C. One ml of this mixture was diluted to 10 ml with 50 Mm MES
pH 6.0 and applied to a Fast S cation exchange column (1.0 ml bed volume)

20   equilibrated in the same buffer. Unconjugated BSA-DM did not bind to the
column. The column was rinsed with starting buffer and bound immunoconjugate
was eluted with 20 Mm phosphate buffer containing 300 Mm NaCl pH 7.2. The
3A3-BSA-DM immunoconjugate was collected in a 1.2 ml fraction.

f.   Cytotoxic Activity of Immunoconjugate 3A3-BSA-DM.

25   Bovine fibronectin (CalBiochem) was diluted to 50 ug/ml in PBS and 0.2
ml aliquots were added to wells of a 24 well culture plate and incubated overnight
at 4°C. Following incubation wells were rinsed twice with PBS to remove
unbound protein. One ml of 3A3-BSA-DM was diluted 1:3 in PBS containing
0.1% BSA and filter sterilized with a 0.45 micron filter and added to wells

30   previously coated fibronectin. Control wells were loaded with PBS containing
0.1% BSA. Following incubation for three hours at 37°C the wells were rinsed
with PBS. One ml of MRC-5 fibroblasts (ATCC No. CCL 171)(1 X 10^4 cells/ml)
in M199 containing 10% FBS was added to each well. 3H-thymidine was added

and the wells processed as described in Example 1d. Results are shown in
Table 5.

<u>Table 5</u>

Cytotoxicity of 3A3-BSA-DM for MRC-5 Fibroblasts

	Incorporation of 3H-Thymidine[1]
3A3-BSA-DM	30,006
0.1% BSA in PBS	107,465

[1]Individual culture wells were coated with solutions of fibronectin and then treated
with 3A3-BSA-DM and PBS containing 0.1% BSA. Following incubation to allow
for immunoconjugate binding, the wells were rinsed with PBS to remove unbound
conjugate and MRC-5 fibroblasts in M199 containing 10% FBS were added.
Following incubation, the ability of cells to incorporated 3H-thymidine was
determined. Data represent average of triplicate samples.

<u>Example 6</u>

<u>Preparation and Assay of Fibronectin-BSA-DM Conjugate</u>

a.   <u>Preparation of Fibronectin-BSA-DM Conjugate</u>.

DM-BSA with free sulfhydryl groups was prepared as described in
Example 5. Bovine plasma fibronectin (0.9 ml) (Calbiochem) at 1.0 mg/ml in
PBS was treated with a 5-fold molar excess of (LC)-SPDP (1 mg/ml in ethanol)
and incubated for 1 hour at room temperature. (LC)-SPDP-fibronectin (0.9 ml)
was mixed with 0.25 ml BSA-DM and incubated for one hour at room temperature
followed by overnight incubation at 4°C to produce Fibronectin-BSA-DM
conjugate.

b.   <u>Cytotoxic Activity of Fibronectin-BSA-DM</u>.

Gelatin (Sigma) was diluted to 100 μg/ml in water and 0.2 ml aliquots
were added to wells of a 24 cell culture plate and allowed to dry overnight at
room temperature. Following incubation, wells were rinsed twice with PBS to
remove unbound material.

Following incubation, 0.5 ml of the Fibronectin-BSA-DM conjugate was
diluted to a fibronectin concentration of 50 ug/ml in PBS containing 0.1% BSA
and added to wells previously coated with gelatin. Control wells were loaded with
PBS containing 0.1% BSA. Following incubation for three hours at 37°C the

32

wells were rinsed with PBS. One ml of MRC-5 fibroblasts (1 X 10⁴ cells/ml) in

M199 containing 10% FBS was added to each well. After incubation for 48

hours, 3H-thymidine was added and the wells processed as described in Example

1d. Results are shown in Table 6. Figure 2 shows a generic schematic

5   representation of the Fibronectin-BSA-DM conjugate.

Table 6

Cytotoxicity of 3A3-BSA-DM for MRC-5 Fibroblasts

	Incorporation of 3H-Thymidine[1]
Fibronectin-BSA-DM	12,255 cpm
0.1% BSA in PBS	135,198 cpm

[1]Individual culture wells were coated with solutions of gelatin and then treated with
Fibronectin-BSA-DM or PBS containing 0.1% BSA. Following incubation to allow
for the conjugate to bind, the wells were rinsed with PBS to remove unbound
conjugate and MRC-5 fibroblasts in M199 containing 10% FBS were added.
Following incubation, the ability of cells to incorporated 3H-thymidine was
determined. Data represent average of triplicate samples.

Example 7

Effect of Fibronectin-BSA-DM on IOP after Filtering
Surgery in Normotensive Rabbits

The creation of a functioning filtering bleb in six 2.0-2.5 kg rabbits is

25   carried out according to the methods described by Lee, *et al.* (Ophthalmol.

94:1523, 1987). The animals receive surgery in both eyes, with one eye treated

with Fibronectin-BSA-DM conjugate and the fellow eye treated with vehicle

control. Rabbits will be given a preoperative eye examination with a Zeiss slit

lamp biomicroscope. Preoperative IOP will be obtained from the average of three

30   measurements by pneunototometry, using an Applanation Pneumatograph

(BioRad), after the installation of one drop of 0.5% proparacaine Hcl to each eye.

Each rabbit receives a subconjunctival injection at the operative site in one eye of

25 ug conjugate in 0.1 ml PBS two hours prior to surgery. The other eye receives

a similar injection of 0.1 ml PBS as a control. The rabbit is placed under general

35   anesthesia, a lid speculum placed, a limbal-based conjunctival portame is made

approximately 8 mm posterior to the limbus and sharp and blunt dissection

performed until the cornea scleral limbus is well visualized. A triangular partial
thickness scleral flap is then developed based at the limbus to approximately 50%
scleral depth, and then an entry wound into the anterior chamber is made with a
sharp 15 degree razor knife. A 1 mm x 3 mm sclerostomy is then performed to
5   excise the tissue under the partial thickness flap. A peripheral iridectomy is
performed with Vannus scissors and curved jewelers' forceps. The flap is sutured
in place with 10-0 nylon suture and the conjunctiva closed with a running
absorbable suture.

The animals are then given a combination antibiotic/steroid ointment
10   applied to each eye, are kept warm, and observed every hour for eight hours and
then every four hours the following day. At 24 and 72 hours post-surgery, the
rabbits are given subconjunctival injections in both eyes as above. Daily
observations continue thereafter with the instillation of the antibiotic steroid
ointment daily for approximately 21 days. Examinations including a routine
15   ophthalmic examination for bleb patency, toxicity and complications, slit lamp
biomicroscopy and pneumotonometry, are performed daily for the first five days
after surgery, then every third day for eight weeks. Observations, including
variations in IOP, are subjected to standard statistical analysis to look for
maintenance of lowered IOP.
20

Example 8

Effect of 3A3-BSA-DM Conjugate on IOP after Filtering
Surgery in Beagles with Glaucoma

Beagle dogs are naturally susceptible to glaucoma and are well accepted as
25   a model for human glaucoma (King et al. (1991) Am. J. Vet. Res. 52:2067-2070).
Two groups of six beagles each with glaucoma, as characterized by IOP greater
than 30 mm Hg in one or both eyes receive glaucoma filtering surgery in one
glaucomatous eye; one group receives 3A3-BSA-DM conjugate and the other
group is treated with vehicle control. Beagles will be given a preoperative eye
30   examination with a Zeiss slit lamp biomicroscope. Preoperative IOP will be
obtained from the average of three measurements by pneunototometry, using an
Applanation Pneumatograph (BioRad), after the installation of one drop of 0.5%
proparacaine HCl to each eye. Each dog receives a subconjunctival injection at

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the operative site in one eye of 25 ug conjugate in 0.1 ml PBS two hours prior to surgery. The other eye receives a similar injection of 0.1 ml PBS as a control. The dog is placed under general anesthesia, a lid speculum placed, a limbal-based conjunctival portame is made approximately 8 mm posterior to the limbus and

5   sharp and blunt dissection performed until the cornea scleral limbus is well visualized. A triangular partial thickness scleral flap is then developed based at the limbus to approximately 50% scleral depth, and then an entry wound into the anterior chamber is made with a sharp 15 degree razor knife. A 1 mm x 3 mm sclerostomy is then performed to excise the tissue under the partial thickness flap.

10   A peripheral iridectomy is performed with Vannus scissors and curved jewelers' forceps. The flap is sutured in place with 10-0 nylon suture and the conjunctiva closed with a running absorbable suture.

The animals are then given a combination antibiotic/steroid ointment applied to each eye, are kept warm, and observed every hour for eight hours and

15   then every four hours the following day. At 24 and 72 hours post-surgery, the beagles are given subconjunctival injections in both eyes as above. Daily observations continue thereafter with the instillation of the antibiotic steroid ointment daily for approximately 21 days. Examinations including a routine ophthalmic examination for bleb patency, toxicity and complications, slit lamp

20   biomicroscopy and pneumotonometry, are performed daily for the first five days after surgery, then every third day for eight weeks. Observations, including variations in IOP, are subjected to standard statistical analysis to look for maintenance of lowered IOP.

25   Example 9
                        Effect of 1B4-RA Immunoconjugate on Development of PVR after
                                            Vitrectomy in Rabbits
                The refined experimental PVR model involving vitreous gas compression
        and gas-fluid exchange as described by Chandler et al., (1986) is used to test in

30   vivo activity efficacy of 1B4-RA immunotoxin in rabbits; i.e., to test the ability of the compound to bind in vivo as well as its ability to exert a cytotoxic effect in vivo. Two groups of six Dutch-Belted rabbits each weighing 1.5-2.0 kg are used in the study. All rabbits undergo vitreous gas compression and gas-fluid

                                                  35

exchange, and one group receives the immunotoxin, while the other receives a
vehicle control. Before each step in the procedure, rabbits are anesthetized with
intramuscular injections of ketamine Hcl (30 mg/kg) and eylazine (5 mg/kg). The
pupils of experimental eyes are dilated with phenylephrine (5.0%) and tropicamide

5   (0.25%). Briefly, cryopexy is performed at approximately 4 mm from the limbus
at the lower nasal quadrant. Seven to 10 days following cryopexy, 0.3 ml of
perfluorocarbone gas is injected intravitreally with a 30-gauge tuberculin syringe in
two portions of 0.2 ml and 0.1 ml. Injections are performed under indirect
ophthalmoscopic observation. The gas is left in the eye for 2 days after which it

10   is replaced with BSS. Seven to 10 days after gas fluid exchange, eyes are
examined for indications of cataract, vitreous hemorrhage, or any detachment.
Animals with any sign of these conditions are eliminated from the study.
Experimental eyes are injected with 25,000 fibroblasts directly above the optic
disc. Cultured rabbit fibroblasts (described above) are harvested from cell culture

15   by trypsinization and resuspended in BSS to achieve a final cell density of
25,000/0.1 ml for intravitreal injection.

Twenty-four hours after injection of the fibroblasts, one group of rabbits
receives an intravitreal injection of 25 ug 1B4-RA in 0.1 ml PBS. The other
group receives an intravitreal injection of 0.1 ml PBS. Eyes are examined by

20   indirect ophthalmoscopy on days 1, 3, 7, 14, and 28 after injection. Appearance
of the retina and fundus are documented by fundus photography. The toxic
changes observed are graded in five stages according to their ophthalmoscopic
appearance: stage 0=clear fundus; stage 1 = gray retinal area near the optic disc;
stage 2= retina attached, but decreased vascularity and small rays; stage 3=retinal

25   detachment without tear; stage 4=retinal detachment with tear. At the end of the
study, the animals are sacrificed and the eyes enucleated. Portions of the posterior
fundus are fixed, processed and embedded for histological examination. Activity
of the immunotoxin is determined by comparing the results from the control and
the treated groups.

30

Example 10
Conjugation of Daunomycin to Carrier Proteins:
Preparation of DM-BSA-DPDPB

Forty mg of 1-ethyl-3-dimethylaminopropylcarbodiimide (EDC) (Pierce)

5   and 80 mg N-hydroxysulfosuccinimide (sulfo-NHS) (Pierce) were added to 1 ml of

bovine serum albumin (BSA) (Sigma) dissolved in 2-(N-Morpholino)ethanesulfonic

acid buffer (MES), (10 mg/ml in 50 mM MES pH 6.0) and incubated for one

minute. Five mg of daunomycin in 0.2 ml of water was added to this solution

followed by incubation for 2 hours at room temperature and overnight at 4°C.

10   The reaction mixture was centrifuged (3000 x g, 30 minutes) to remove any

precipitation formed during the reaction and the supernatant containing the BSA-

DM conjugate was desalted over a Econo-Pac10DG column (Bio Rad) (10 ml bed

volume) equilibrated with 50 mM Tris buffer pH 8.0. Dithiothreitol (DTT) was

added to give a final concentration of 50 mM followed by incubation at room

15   temperature for 50 minutes. The reduced BSA-DM was desalted over a 1.5 x

32 cm Sephadex G-25 column equilibrated with PBS containing 1 mM EDTA.

One 4-di-3'-(2'pyridyldithio)-propionamido) butane (DPDPB) (Pierce) (0.1 ml at

10 mg/ml in DMSO) was added per ml of BSA-DM and allowed to react for 45

minutes at room temperature. Following incubation, unconjugated reactants were

20   removed by desalting over a 1.5 x 32 cm Sephadex G-25 column equilibrated with

PBS containing 1 mM EDTA. BSA-DM-DPDPB conjugate was used within one

hour for coupling to ECM binding proteins as described below.

Example 11
Conjugation of DM-BSA-DPDPB to 7G12 Fab'

25   Purified 7G12 IgG (2.3 mg/ml, 39 ml) in 0.1 M citrate buffer pH 3.8 was

mixed with pepsin (Sigma, 3700U/mg, 0.9 ml of a 1 mg/ml solution) and

incubated for four hours at 37°C. The reaction was stopped by adjusting the pH

to 7.0 with 3.0 ml of 3M Tris buffer pH 8.0. DTT was added to give a final

30   concentration of 50 mM followed by incubation at 37°C for 30 minutes. The

reduced 7G12 Fab' was isolated by desalting over a 1.5 x 32 cm Sephadex G-25

column equilibrated with PBS containing 1 mM EDTA.

DM-BSA-DPDPB (17 ml containing 0.13 mg daunomycin/ml) was mixed
with 30 ml of reduced 7G12 Fab' (1.4 mg/ml) and incubated for one hour at room
temperature followed by 48 hours at 4°C. Following incubation, the Fab'-BSA-
DM conjugate was centrifuged to remove insoluble precipitates. The supernatant
5   was passed through a 0.45 micron filter and applied to a anion exchange column
(HITrap Q, 2 ml, Pharmacia) equilibrated with PBS. Bound conjugate was eluted
with PBS containing 2 M NaCl. Fractions containing 7G12 Fab'-BSA-DM were
pooled and applied to a 1.5 x 28 cm Sephadex G-25 column equilibrated with
PBS. Fractions containing protein and daunomycin eluting in the column void
10   volume were pooled and stored at -20°C.

Example 12
Conjugation of DM-BSA-DPDPB to the Collagen
Binding Domain of Fibronectin (FnCD)

15   Purified bovine plasma fibronectin (1.0 mg/ml) in 50 mM Tris buffer
pH 8.9 was digested with thermolysin (Sigma, 5 µl of a 1 mg/ml stock solution
per ml of fibronectin) dissolved in 50 mM Tris buffer pH 8.0 for 4 hours at 37°C.
The reaction was stopped by the addition of EDTA to give a final concentration of
50 mM. The digested fibronectin was applied to a 27 ml gelatin sepharose
20   (Pharmacia) column and the column was washed with 50 mM Tris buffer pH 8.0
containing 10 mM EDTA. Collagen binding fragments were eluted from the
column with 4 M urea in 50 mM Tris buffer, pH 8.0 and fractions containing
proteins as determined by absorbance at 280 nm were pooled. Collagen binding
fragments containing the fibronectin collagen binding (FnCD) domain were
25   desalted into 50 mM Tris pH 8.0 using a 5 x 15 cm Sephadex G-25 column and
pooled. FnCD was then applied to a 2 ml HITrap Q column equilibrated with the
same buffer. FnCD fractions were eluted with 50 mM Tris pH 8.0 containing
1 M NaCL, pooled and desalted into 0.2 M sodium phosphate buffer pH 8.0
containing 1 mM EDTA using a 1.5 x 28 cm Sephadex G-25 column. Purified
30   FnCD was treated with a fivefold molar excess of 2-imminothiolane-HCl (2-IT)
(Pierce) and incubated for 60 minutes at 30°C. Following incubation, the
derivatized FnCD was desalted into PBS using 1.5 x32 cm Sephadex G-25 column
equilibrated with PBS containing 1 mM EDTA. FnCD-2IT (15 ml, 4.86 mg/ml)

was mixed with DM-BSA-DPDPB (22.4 ml, 0.28 mg/ml) prepared as described
above. The mixture was incubated 2 hours at room temperature and 48 hours at
4°C. FnCD-BSA-DM was purified by passage over an anion exchange
chromatography (HITrap Q, 5 ml column) equilibrated with PBS. The FnCD-

5   BSA-DM conjugate was eluted from the column using 2 M NaCl in PBS.
Fractions containing protein and daunomycin were pooled and desalted over a
1.5 x 28 cm Sephadex G-25 column and stored at -20°C.

                                    Example 13
10   Conjugation of DM-BSA-DPDPB to
                            Hyaluronic Acid Binding Peptides
        A peptide binding hyaluronic acid (Goetinck, et al., (1987) J. Cell Bio.
105:2403-2408) containing a cysetine at the amino terminal end was synthesized.
The peptide sequence is shown below.

15   H-Cys-Ala-Gly-Trp-Leu-Ser-Asp-Gly-Ser-Val-Gn-Tyr-Pro-Ile-Thr-
         Lys-Pro-Arg-Glu-Pro-OH
        Synthesis was performed on an Applied Biosystems Peptide Synthesizer
Model 431A. The peptide was synthesized using standard solid peptide synthesis
employing Fmoc chemistry. The side chain protecting groups for the amino acids

20   were: t-butyl for Asp, Glu, Ser, Thr, Tyr; trityl for Ala, Cys, Gin, His; Pmo for
Arg; and Boc for Lys. Fmoc amino acids were activated using one equivalent of
0.45M 2-(1H-benzotriazol-1-yl)1,1,3,3-tetraethyl-uronium hexaflurophoshate
(HBTU)/ 1 hydroxybenzotriazole (HOBt) solution and two equivalents of N,N-
diisopropylethylamine (DIEA). Synthesis was started with 2-chlorotrityl resin.

25   Fmoc protecting groups were removed using 25% piperidine/NMP.
        Deprotection and cleavage of the peptide from the resin was performed
using trifluroacetic acid (TFA) in the presence of a scavenger mixture (0.75 g
phenol, 0.25 ml EDT, 0.5 ml thioanisole, 0.5 ml water and 10 ml TFA) for three
hours at room temperature. The peptide was purified to greater than 95% purity

30   by reverse phase HPLC using a C18 column with an AB gradient from 0% B
where A is 0.1% TFA in water and B is 0.08% TFA in acetonitrile.
        Seventeen mg of lyophilized peptide was resuspended in 1 ml of water and
added to 20.0 ml of BSA-DM-DPDPB in PBS containing 1 mM EDTA followed

                                        39

by overnight incubation at 4°C. The conjugate was purified by chromatography over a 1 ml HITrap Q column equilibrated with PBS. The conjugate was eluted from the column using 2 M NaCl in PBS and fractions containing protein and daunomycin were pooled and applied to a 1.5 x 28 cm Sephadex G-25 column

5 equilibrated with PBS. The peptide-BSA-DM conjugate fraction eluting in the column void volume was collected and stored at -20°C.

Example 14

Conjugation of Daunomycin to Hyaluronic Acid Binding

10   Peptides Containing a Terminal Hydrazide Group

A peptide binding hyaluronic acid was synthesized using standard Fmoc solid phase synthesis chemistry starting with Wang resin and using appropriate N-Fmoc-amino acids. Side chain protecting groups were tert-Butyl ethers and esters for Asp, Ser and Glu; Boc for Lys; and trityl group for Asn. Fmoc

15 removal was performed with piperidine-DMF, followed by washing with DMF. Coupling was achieved by combining solid Fmoc-amino acids, HoBt and benzotriazol-1-yl-oly tris (dimethylamino) phosphonium hexafluorophosphate (BOP) and n-methylmorpholine (NMM) under nitrogen at 25°C. Following the addition of the last amino acid residue and deblocking of the terminal NH2 group,

20 the peptide was treated with an excess of succinic anhydride. Following washing, an excess of tert-butyl carbazate (Aldrich) was added and coupled to the terminal carboxyl group using carbodiimide. Protected peptide resin was then dried in vacuum and cleaved with TFA-scavenger mix solvent. After four hours at 25°C, the cleavage mixture was filtered, precipitated with ether, collected by

25 centrifugation and lyophilized. The peptide sequence with the terminal hydrazide group is shown below.

NH_2-NH-C(O)-CH2-CH2-C(O)-NH-Leu-Ala-Leu-Asp-Trp-Cys-Asn-
Ala-Gly-Trp-Leu-Ser-Asp-Gly-Ser-Val-Gn-Tyr-Pro-Ile-Thr-Lys-Pro-
Arg-Glu-Pro-Cys-Gly-NH_2
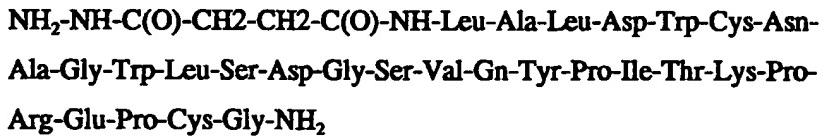
30   Thirty mg of lyophilized peptide was resuspended in 2.0 ml 0.2 M sodium acetate buffer pH 6.0 and added to 0.43 ml of daunomycin (28 mg/ml) followed by overnight incubation at 4°C. DTT was added to a final concentration of 50 mM followed by incubation at room temperature for 2 hours. The conjugate

was centrifuged, passed through a 0.45 micron filter and purified by
chromatography on a 1.5 x 15 cm Sephadex G-10 column equilibrated with PBS.
The peptide-Hz-DM conjugate fraction eluted in the column void volume while
free daunomycin came off the column considerably later.

5

Example 15
Conjugation of Daunomycin to BSA-Hydrazide

a.   Preparation of BSA-Hz Coupled to Daunomycin.

BSA containing reactive hydrazide groups (BSA-Hz) was prepared as
10   follows. To 250 mg BSA in 34.4 ml of water was added 3 g adipic acid
dihydrazide (Sigma) with stirring. The pH was adjusted to 4.75 with 1 N HCl and
90 mg EDC were added. The mixture was allowed to react for one hour at room
temperature with stirring while maintaining pH at 4.75. Following reaction, the
solution was dialyzed at 4°C against distilled water to remove unreacted
15   dihydrazide and EDC and stored at 4°C.

BSA-Hz (0.5 ml, 3.8 mg/ml in 0.2 M sodium acetate buffer pH 6.0) was
mixed with 0.187 mg daunomycin in 7.5 μl of water. The mixture was incubated
overnight at room temperature followed by chromatography over a Econo
Pac10DG column equilibrated with 0.2 M sodium acetate pH 6.0. The BSA-Hz-
20   DM conjugate fraction eluted in the column void volume while free daunomycin
came off the column considerably later. The concentration of daunomycin in the
conjugate fraction was determined spectrophotometrically. The molecular
substitution ratio was 12 molecules of daunomycin per molecule of BSA.

b.   Preparation of Peptides Derived from BSA-Hz Coupled to Daunomycin.

25   Three ml of BSA-Hz (3.9 mg/ml in 0.1 M sodium citrate buffer pH 3.8)
was mixed with pepsin (Sigma) (1.0 mg/ml stock in citrate buffer) to give a 2%
(w/w) final pepsin concentration. The mixture was incubated overnight at 37°C
and the reaction stopped by the addition of 0.75 ml 1 M Tris buffer pH 8.0. Two
ml of this preparation was desalted over a 1.5 x 15 cm Sephadex G-25 column
30   equilibrated with 0.1 M sodium acetate buffer pH 6.0. To 2.5 ml of the desalted
BSA peptide-Hz mixture was added 3 mg daunomycin in 0.12 ml water followed
by incubation at room temperature overnight. BSA peptide-Hz-DM was separated
from free DM by chromatography over a 1.5 x 15 cm Sephadex G-10 column

equilibrated with PBS. The BSA peptide-Hz-DM conjugate fraction eluted in the
column void volume while free daunomycin came off the column considerably
later.

5   Example 16

                        Conjugation of 7G12 to BSA Peptide-Hz-DM

             Purified 7G12 IgG in PBS (5 ml at 2.0 mg/ml) is treated with a fivefold
       molar excess of N-succinimidyl-32-pyridyldithiopropionate (SPDP) (1 mg/ml stock
       solution in absolute ethanol) with rapid mixing. Following incubation for 30
10   minutes at room temperature, the derivatized IgG is separated from unreacted
       SPDP by chromatography over a 1.5 x 40 cm Sephadex G-25 column equilibrated
       with PBS. Fractions containing protein as determined by absorbance at 280 nm
       are pooled and stored at 4°C. BSA peptide Hz-DM (2 mg protein/ml. 5 ml) in
       PBS is treated with DTT to give a final 50 mM concentration followed by
15   incubation at room temperature for 30 minutes. The reduced conjugate is
       separated from DTT by passage over a 1.5 x 50 cm Sephadex G-10 column. The
       conjugate is eluted from the column in the void volume and the protein and
       daunomycin containing fractions are pooled. Five ml of reduced BSA peptide-Hz-
       DM conjugate is mixed with 5 ml of SPDP derivatized IgG and incubated at room
20   temperature for one hour followed by incubation at 4°C overnight. The
       7G12-BSA peptide -DM conjugate is then purified by chromatography over a
       1.5 x 120 cm Sephadex G-50 column equilibrated with PBS. Fractions eluting in
       the column void column containing protein and daunomycin are collected, pooled
       and stored at -20°C.
25

             It is evident from the above results, that modulation of wound healing can
       be obtained using the conjugates of the subject invention. It is found that one can
       use conjugates of antibodies that target particular components of the extracellular
       matrix of the sclera and/or the conjunctiva, or of the vitreous coupled to an
30   antiproliferative agent to inhibit proliferation of cells into a surgical wound site
       such as is introduced during glaucoma filtering surgery or as a result of trauma to
       the eye as is observed in PVR.

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All publications and patent applications mentioned in the specification are
indicative of the level of skill of those skilled in the art to which this invention
pertains. All publications and patent applications are herein incorporated by
reference to the same extent as if each individual publication or patent application
5   was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way
of illustration and example for purposes of clarity of understanding, it would be
obvio us that certain changes and modifications may be practiced within the scope
10   of independent claims.

What is claimed is:

1.   A method for modulating wound healing in a tissue of a mammalian host, said method comprising:

contacting said tissue with a conjugate comprising a cell

5   proliferation modulating agent and a first member of a specific binding
pair, wherein said first member binds with high affinity to a second
member of said specific binding pair, and wherein said second member is a
binding site for said first member on a component of an extracellular
matrix in said tissue.

10

2.   The method according to Claim 1, wherein said component is a
collagen, a proteoglycan or a fibronectin.

3.   The method according to Claim 2, wherein said tissue is sclera or
15   conjunctiva.

4.   The method according to Claim 3, wherein said tissue is an
epiretinal membrane.

20   5.   The method according to Claim 1, wherein said modulating is
inhibiting.

6.   The method according to Claim 5, wherein said cell proliferation
modulating agent is an antimetabolite or an antimitotic.

25

7.   The method according to Claim 6, wherein said antimitotic is
daunomycin.

8.   The method according to Claim 5, wherein said cell proliferation
30   modulating agent is a toxin or a toxic portion of a toxin.

9.   The method according to Claim 8, wherein said toxin is ricin.

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10.   The method according to Claim 1, wherein said first member is an antibody.

11.   The method according to Claim 10, wherein said antibody is a
5   monoclonal antibody.

12.   The method according to Claim 10, wherein said antibody is a bifunctional antibody.

10   13.   The method according to Claim 11, wherein said first member comprises a binding domain of a polypeptide.

14.   The method according to Claim 13, wherein said component is a collagen.
15

15.   The method according to Claim 14, wherein said polypeptide is fibronectin.

16.   The method according to Claim 1, wherein said cell proliferation
20   modulating agent and said first member are conjoined by an acid labile linking group, a linking group which is cleaved by an enzyme endogenous to said tissue, or a group which is reduced by a reducing agent.

17.   The method according to Claim 1, wherein said conjugate further
25   comprises a protein carrier interposed between said cell proliferation modulating agent and said first member.

18.   The method according to Claim 17, wherein said protein carrier is serum albumin.
30

19.   The method according to Claim 18, wherein said cell proliferation modulating agent comprises 10 to 30 molecules of agent per molecule of said carrier protein.

20.   The method according to Claim 1, wherein said wound is a fistula.

21.   The method according to Claim 1, wherein said component is
hyaluronic acid.

5   22.   The method according to Claim 21, wherein said first member is
hyaluronan binding protein.

23.   The method according to Claim 22, wherein said hyaluronan
binding protein is a link protein.

10

24.   A method for inhibiting healing of a fistula, said method
comprising:
               contacting tissue abutting said fistula with a conjugate comprising a
cytotoxic agent and a first member of a specific binding pair which binds with
15   high affinity to a second member of said specific binding pair, wherein said first
member comprises an antibody or a macromolecule comprising a binding domain
of a polypeptide, and wherein said second member is a binding site for said first
member on a component of an extracellular matrix in said tissue.

20   25.   The method according to Claim 25, wherein said tissue is sclera,
conjunctiva, or an epiretinal membrane.

26.   The method according to Claim 25, wherein said component is a
collagen.

25

27.   The method according to Claim 25, wherein said polypeptide is a
fibronectin.

28.   The method according to Claim 25, wherein said first member is an
30   antibody.

29.   The method according to Claim 25, wherein said cytotoxic agent is
an antimetabolite or an antimitotic.

46

30.   The method according to Claim 30, wherein said antimitotic is daunomycin.

31.   The method according to Claim 25, wherein said cytotoxic agent is a ricin A chain.

32.   The method according to Claim 25, wherein said conjugate is in a physiologically acceptable carrier.

33.   A method for inhibiting growth of cells migrating into the extracellular matrix of tissue abutting a fistula in ocular tissue, said method comprising:

        contacting said tissue with a conjugate comprising an antiproliferative agent and a macromolecule which binds with high affinity to a component of said extracellular matrix.

34.   The method according to Claim 33, wherein said antiproliferative agent is daunomycin.

35.   The method according to Claim 33, wherein said component is a collagen, a fibronectin, or a proteoglycan.

36.   The method according to Claim 33, wherein said macromolecule comprises an antibody or a fragment thereof.

37.   The method according to Claim 33, wherein said macromolecule comprises a collagen-binding domain obtainable from a fibronectin.

38.   A method for maintaining a fistula in ocular tissue in a mammal, said method comprising:

        creating a fistula site in said ocular tissue; and contacting tissue abutting said fistula site with a conjugate comprising daunomycin and a

47

macromolecule which comprises a domain which binds with high affinity to a component of an extracellular matrix of said tissue.

39.   The method according to Claim 38, wherein said component is a collagen, a fibronectin, or a proteoglycan

40.   The method according to Claim 38, wherein said macromolecule comprises an antibody or a collagen-binding domain obtainable from fibronectin.

41.   A composition comprising:
       a conjugate comprising a cytotoxic agent and a macromolecule which comprises a first domain which binds with high affinity to a component of an extracellular matrix in ocular tissue.

42.   The method according to Claim 41, wherein said ocular tissue is sclera, conjunctiva or an epiretinal membrane.

43.   The composition according to Claim 42, wherein said cytotoxic agent is daunomycin.

44.   The composition according to Claim 42, wherein said cytotoxic agent comprises Ricin A chain.

45.   The composition according to Claim 41, wherein said component is a collagen, a proteoglycan or a fibronectin

46.   The composition according to Claim 41, wherein said macromolecule further comprises a second domain which binds with high affinity to a cell in said ocular tissue.

47.   The composition according to Claim 46, wherein said cell is a fibroblast.

48.   The composition according to Claim 47, wherein said cytoxic agent comprises ricin A chain toxin.

49.   The composition according to Claim 47, wherein said
5   macromolecule is an antibody.

50.   A composition comprising:
       a conjugate comprising daunomycin and a first macromolecule
which comprises a domain which binds with high affinity to a collagen, a
10   fibronectin, or a proteoglycan.

51.   The composition according to Claim 50, wherein said first
macromolecule comprises an antibody, a fragment of an antibody, or a binding
domain obtainable from a polypeptide.
15

52.   The composition according to Claim 51, wherein said antibody is a
monoclonal antibody.

53.   The composition according to Claim 51, wherein said polypeptide is
20   fibronectin.

54.   The composition according to Claim 50, wherein said conjugate
further comprises a carrier molecule comprising a second macromolecule between
said daunomycin and said first macromolecule.
25

55.   The composition according to Claim 54, wherein said second
macromolecule comprises human serum albumin.

56.   The composition according to Claim 50, wherein said conjugate is
30   reversibly bound to a solid support.

57.   A composition comprising:

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a conjugate comprising cytotoxic agent and a bifunctional antibody which comprises a first domain which binds with high affinity to a collagen and a second domain which binds with high affinity to a fibroblast.

5   58.   The composition according to Claim 57, wherein said cytotoxic agent is daunomycin.

           59.   The composition according to Claim 58, wherein said conjugate further comprises human serum albumin as a carrier molecule between said
10   daunomycin and said bifunctional antibody.

           60.   The composition according to Claim 57, wherein said cytotoxic agent comprises ricin A chain toxin.

15   61.   The composition according to Claim 58 or 60, wherein said bifunctional antibody is a bifunctional monoclonal antibody.

Figure 1

Fibroblast
17

EXTRACELLULAR MATRIX (ECM) COMPONENTS OF SCLERA AND CONJUNCTIVA

Cytotoxic Agent 15

ECM BINDING PROTEIN

11

13
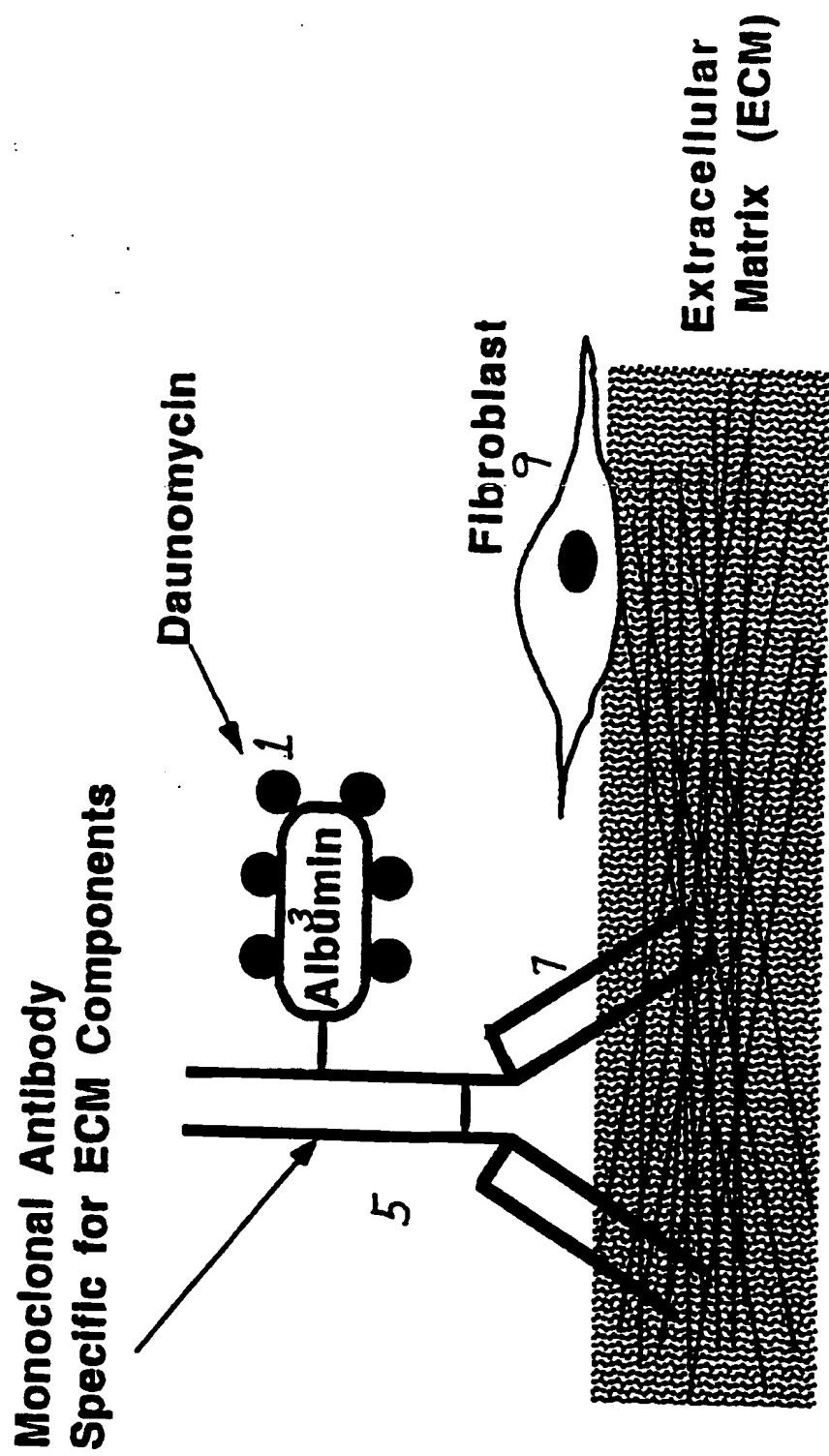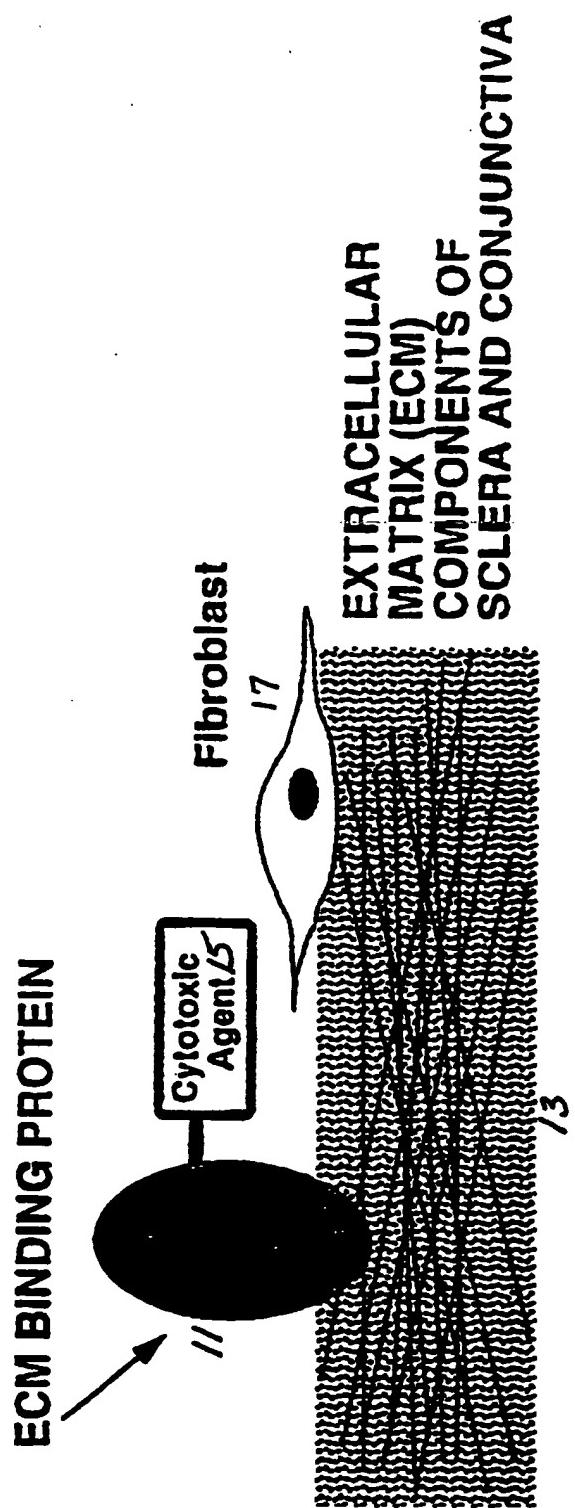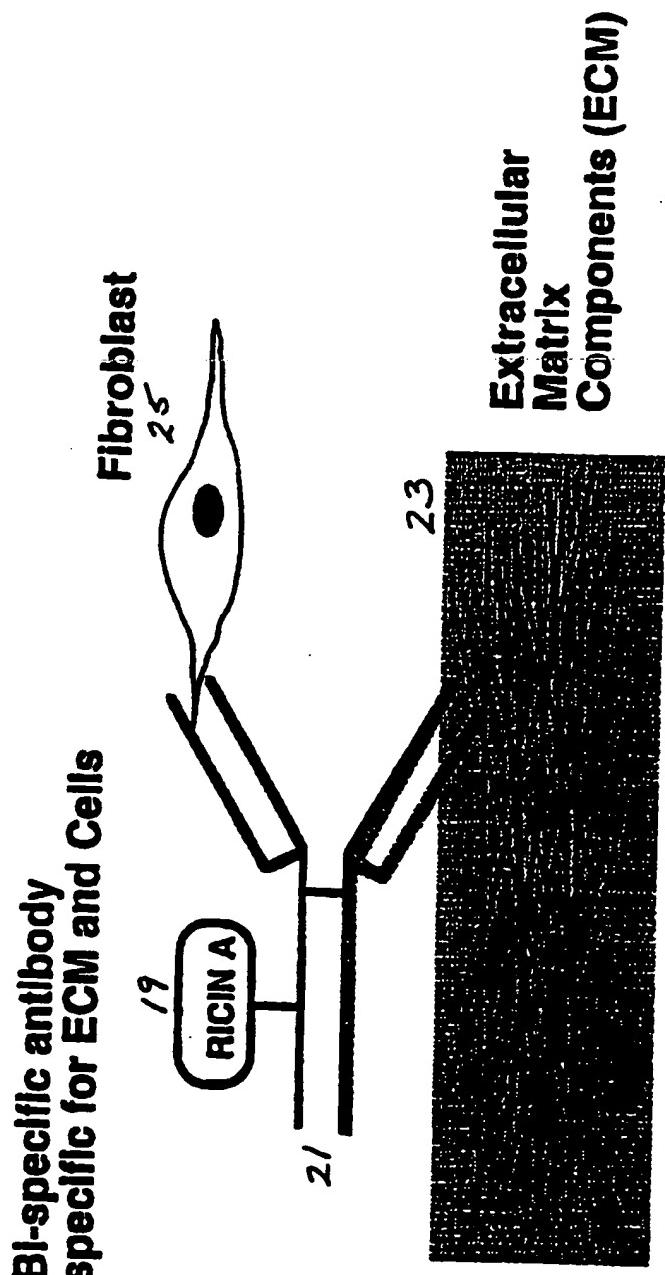
Figure 2

Figure 3

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,89 12093 (BAYLOR COLLEGE MEDICINE) 14 December 1989 see page 3, line 6 – line 27 ---	1,8-11, 16,41, 42,44
P,X	WO,A,95 03828 (HOUSTON BIOTECHNOLOGY) 9 February 1995 see page 5, line 8 – page 6, line 6; claims 1,2,4; example 3; table 3 --- -/--	1,8,41, 44

X	Further documents are listed in the continuation of box C.		X	Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 October 1995	27. 10. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Berte, M

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 160 521 (GREEN CROSS CORP) 6 November 1985	1-7,13, 15,16, 19,24, 25,27, 29,30, 32-35, 41-43, 45-47, 50,53,54
Y	see page 1, paragraph 1; claims 1,3,6,9 see page 3, line 6 - line 19 see page 5, line 4 - line 8; table 1	1-61
X	EP,A,0 114 685 (GREEN CROSS CORP) 1 August 1984 see page 3, line 9 - line 23 see page 4, line 23 - page 5, line 25	1,2,6,7, 13,15
X	WO,A,92 12739 (UNIV LONDON) 6 August 1992 see page 1, line 5 - line 17 see page 2, line 11 - line 24 see page 3, line 20 - line 26 see page 7, line 1 - line 16 see page 12, line 9 - line 24	1
Y	see page 18, line 1 - line 8; claims 1-4,6	1-61

International application No.

PCT/US95/07395

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 1-40 are directed to a method of treatment of (dia-

gnostic method practised on) the human/animal body the search has been car-
ried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.:   1-61
because they relate to parts of the international application that do not comply with the prescribed requirements to such
an extent that no meaningful international search can be carried out, specifically:

please see enclosed sheet./.

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all
searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report
covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   [ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

INTERNATIONAL SEARCH REPORT

FURTHER INFORMATION CONTINUED FROM   PCT/ISA/210

INCOMPLETE SEARCH
2. OBSCURITIES,......etc.
In view of the large number of compounds which are designed by the compounds
mentioned in claims the search had to be restricted for economic reasons.
The search was limited to the compounds for which pharmacological data was
given and/or the compounds mentioned in the claims or examples (see guide-
lines, part B, chapter III, paragraph 3.6)

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-8912093	14-12-89	US-A-	4871350	03-10-89
		US-A-	5202252	13-04-93
		AT-T-	126530	15-09-95
		AU-B-	623666	21-05-92
		AU-B-	3850989	05-01-90
		DE-D-	68923852	21-09-95
		EP-A-	0372071	13-06-90
		FI-B-	94258	28-04-95
		FI-C-	94258	10-08-95
		JP-T-	3501806	25-04-91
		US-A-	5055291	08-10-91
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		IE-B-	62618	22-02-95
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		CA-A-	1247010	20-12-88
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		CA-A-	1223202	23-06-87
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		AU-B-	1166692	27-08-92
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		EP-A-	0567528	03-11-93
		JP-T-	6504546	26-05-94